Wakeham Book III

(84)

MEETING

SCIENTIFIC ADVISORY BOARD

September 10, 11, 12, 1975

Georgetown University

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BOOK No. III

Applications from Georgetown University School of Medicine and Dentistry

CONTENTS:

1. Program Project:

   "Smoking and Lung Development"
   Principal Investigator -- Paul Hamosh, M.D.
   New Application No. 1049

2. "Effects of Cigarette Smoke Exposure on Developmental, Cellular and Molecular Aspects of the Immune Response"
   Principal Investigator -- Herbert B. Herscowitz, Ph.D.
   New Application No. 1045

3. For informational purposes:

   "The Effect of Cigarette Smoke on Lung Metabolism"
   Principal Investigator -- Branislav Vidic, S.D.
   Second Renewal Application No. 936R2
   (This renewal will be handled administratively in the customary manner.)

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LOMA-HAMOSH

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THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

July 23, 1975

Grant Application No. 1049 (Program Project)

PULMONARY

To: The committee comprising Drs. Feldman, Gardner, Jacobson and Sommers.

Subject: Paul Hamosh, Ph.D., Georgetown University, Washington, D.C.
New application No. 1049
"Smoking and Lung Development: A Program Project"

History

In the past, CTR has supported Dr. Hamosh, Vidic and Herscowitz at the Georgetown University: more recently, within the newly established Center for Interdisciplinary Studies in Immunology. Georgetown University was awarded a Pulmonary Program Project by NHLI in 1974.

Dr. Herscowitz has been supported by CTR since January 1973: Dr. Hamosh since January 1973, and Dr. Vidic since January 1974.

The present application represents the cooperative spirit of the three CTR grantee members and the Center: takes advantage of the back-up efforts of the studies by Drs. Herscowitz and Vidic.

The concept of a Program Project application was encouraged by CTR after an informal resource site-visit by Drs. Gardner, Hockett, Lisanti and Stone, at which time the potential was both formally and informally presented by the Medical Center staff and the various Directors of special interest.

Request -

Application No. 1049 requests a total of $275,495 for the first year of a five year program.

Dr. Hamosh's present grant (No. 878R2) "Effects of Smoking on the Small Airways", supported at a level of $28,265, will terminate December 31, 1975.

Documents Submitted (attached)

1. Program Application dated June 30, 1975.

This includes:

(a) Overview of Program -- pages 1 - 14
Total Program Budget (page 10)

(b) Project I -- pages 15 - 38 (including Project I budget (page 38)

(c) Project 2 -- pages 39 - 54 (including Project 2 budget (page 54)

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(d) Project 3 -- pages 59 - 71 (including Project 3 budget (page 71)

(e) Facilities Available (page 72 -74)

(f) Justification of Budget (page 75 -76)

(g) Biographical Sketches (pages 77 - 92)

(h) Other Financial Support (page 93)

2. Appendix 1 (1 page) and Appendix 2 (24 pages)

3. Addenda:

(a) Addendum I -- a Comprehensive Report on Achievements of the "Group" (12 pages)

(b) Addendum 2 -- Human Research Consent Forms
(a) From the University-unsigned
(b) Sample of Parent Consent Form

(c) See Comments

4. 19 assorted reprints.

Comments

1. Addendum 2 -- Respiratory Questionnaires -- statistical work-up and descriptions of electronics for Project 1A

and

Addendum 3 -- Description of morphometry, including Figure 7 will be forwarded to you as soon as received by CTR.

2. Dr. P. Hamosh salary:

P. Hamosh will devote 60 percent of his time to the Program: however, only $12,427 (ie. 35 percent of his salary) is requested from CTR -- the remaining 25 percent will be provided from other sources.

David Stone

DS/lp
Encls.

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Received: July 7 1975
Application No. 1049

# SMOKING AND LUNG DEVELOPMENT: A PROGRAM PROJECT

JUL 7 - 1975

RESPONSIBLE INVESTIGATOR: PAUL HAMOSH M.D.,
ASSOCIATE PROFESSOR OF PHYSIOLOGY,
BIOPHYSICS AND MEDICINE

SUBMITTED TO THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

FROM THE GEORGETOWN UNIVERSITY CENTER FOR
INTERDISCIPLINARY STUDIES IN IMMUNOLOGY AND
THE DEPARTMENTS OF PHYSIOLOGY AND BIOPHYSICS
AND ANATOMY

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# THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: 6/30/75

1. Principal Investigator (give title and degrees):

Paul Hamosh, M.D., Associate Professor of Physiology, Biophysics and Medicine

2. Institution & address:

Georgetown University
37th & O Streets, N.W.
Washington, D.C. 20007

3. Department(s) where research will be done or collaboration provided:

Departments of Physiology and Biophysics, Anatomy, Microbiology and the Georgetown University Center for the Interdisciplinary Studies in Immunology

4. Short title of study:

SMOKING AND LUNG DEVELOPMENT: A PROGRAM PROJECT

5. Proposed starting date: 1/1/76

6. Estimated time to complete: 5 years

7. Brief description of specific research aims:

An integrated, interdisciplinary approach to study the relationship between lung structure and lung function (physiological and biochemical) based on the assumption that hereditary, congenital and/or acquired (early in life) differences in geometrical patterns of the airways and lung parenchyma might be the major predisposing factor in environmental lung disease.

Three projects with major emphasis on physiology and pathology, biochemistry and anatomy (cellular and embryological) respectively, will have the following principal aims:

In Project 1, we will study the A) correlation between teenage smoking and the maturation of lung function.B) the correlation between smoking and morphometry of the lung in adolescent and young accident victims. C) The effect of mechanical stress on the pathogenesis of lung disease in experimental animals. D) the effect of smoking a single cigarette as a possible "provocation test" to separate "reactors" from "non-reactors".

In Project 2, we shall study the development of biochemical composition and function in the maturing experimental animals and the effect of smoking on biochemical development.

In Project 3, we shall study the effect of smoking on the isolated lung cells and work with genetic and "manipulated" variants with different geometrical patterns of the airways. (An abstract of all projects is on Page 5).

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OVERVIEW

P. HAMOSH M.D.

PROGRAM PROJECT

SMOKING AND LUNG DEVELOPMENT

RESPONSIBLE INVESTIGATOR: Paul Hamosh, M.D.

Historical Background. Georgetown University has recently approved the establishment of a Center for Interdisciplinary Studies in Immunology (CENTER) under the direction of Dr. Joseph Bellanti, Professor of Pediatrics and Microbiology (DIRECTOR OF CENTER). The aim of this Center is to perform coordinated research, training and clinical service with the co-operation of members, professionalsfrom different subspecialties who are distributed in the various departments of the Medical School. The nucleus for the research activities of the Center is a Pulmonary Program Project (PPP) awarded by NHLI in 1974, (HL16748). This Center has attracted many investigators, among them Drs. Hamosh, Herscowitz and Vidic, who are also grantees of the Council for Tobacco Research, Inc., U.S.A. (CTR). This resulted in gradual tightening of cooperation between these investigators with sharing of intellectual (common projects) and material resources (manpower, supplies, and equipment).

The purpose of the present program project is to expand the capabilities of the Center to achieve its goal of attracting more senior and junior investigators in the pursuit of excellence in research. The organizational interaction of all ongoing and planned functions of the Center is illustrated on Figure 1.

The proposed program project encompasses a number of projects and subprojects all tied around a central theme: Smoking and Lung Development. The working hypothesis is as follows:

Hereditary, congenital, or acquired (during maturation) factors influence profoundly lung growth and maturation. This results in a variety of geometrical patterns of lung airways and parenchyma. The geometrical structure of the lung determines its aerodynamic properties. It may well be that certain patterns of lung structure, while "physiologically" normal, may predispose to certain diseases, particularly when associated with environmental factors such as cigarette smoking and air pollution. Identifying subjects with such "predisposing lung patterns" might greatly aid in the prediction of response of the individual to environmental hazards.

The program project will be under the direction of the responsible investigator (RI) and is subdivided into three major projects centering around the physiological, biochemical and anatomical aspects of the basic theme. Each project has its principal investigator and co-investigators who are participating in several projects. The organization of the program project is illustrated on Figure 2.

Most of the projects in this program are based on previous and



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present investigations funded by the CTR and to some extent by other agencies. This program is the logical outgrowth of an evolution in thought based on over two years of close work among the participating investigators. The support of the CTR, therefore, was "seed money" for further growth.

The mechanisms of concerted action will be provided by weekly meetings of the participating investigators and by pooling of information derived in these studies and from the literature. A pool of consultants will also be available. The Center provides the framework through which additional resources can be brought to play. This is especially true, considering the training and service branches, which can be tapped for additional manpower and clinical material.

The participating investigators span several departments, including Anatomy, Physiology, Pathology, Microbiology, Pediatrics and Medicine. The resources of these departments are also available for the purpose of this program project. The animal facilities of the University are fully accredited and the computational facilities are available by the courtesy of the "National Biomedical Research Foundation", a nonprofit organization affiliated with the Department of Physiology. All facilities used in this program are concentrated in two adjacent buildings, which facilitates geographically the co-operative venture (Figure 3).

The proposed program project is supplemental to the Pulmonary Programs Project which is immunologically oriented, but also emphasizes development and maturation of immuno-competence. The future cross-fertilization of these two program projects will increase the cohesiveness of the Center and establish a mechanism for attracting excellent young people for training and research.

Collaborative Arrangements

A. Within The Program Project

Each of the investigators is an expert in his field and directs a laboratory in his field. Each has (or will have) technical assistants trained to perform specific procedures. Most of the procedures described in Projects 1B,C and D, Project 2 and some in Project 3 have been performed before by the investigators. Usually a series of experiments are planned in advance, a time table prepared. Physiological measurements are performed first after which the specimens are distributed by the experimenter to the Biochemical and Morphology laboratory. Due to short distances and good communication, co-operation is proceeding smoothly.

B. Within The Center

The resources of the center are available to all investigators. At this time many of the technical personnel, paid from different sources

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perform regular work for projects funded by the CTR. Of course, this is a two-way arrangement, but pooling of resources was one of the aims of the establishment of the Center.

C. Within The University

The Center and the Program Project have the support of the Departmental Chairmen, who govern the Medical School out of the Executive Faculty. The Director of the Center and the Responsible Investigator of the Program Project are tenured faculty in the Medical School. The administration of the research is enhanced by the Office of Sponsored Programs, specially instituted in 1970 to assist investigators with extra-mural support. Research on human subjects is reviewed and approved by the Research Committee and Clinical Study Committee.

D. Outside The University

Numerous collaborative arrangements are active now between the investigators in this program and investigators at other institutes in the area. To name a few: the Responsible Investigator is a part-time employee in oncology research at the local Veterans Administration Hospital. M. Hamosh worked for nine years in the National Institute of Health and as a result of it, numerous resources of the Institute are available to the group and free flow of specimens is going both ways.

A number of outside consultants will participate in advisory capacities in the Program Project. Because of technical reasons few could be contacted so far. An addendum will be sent in within the next two months with a list. Tentatively they are: Solbert Permutt, M.D. (Project 1A), Jerome Kleinerman, M.D. (1B), Donald L. Fry M.D. (1C) and Phillip M. Farrell, M.D. (Project 2).



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OVERVIEW

P. HAMOSH M.D.

ABSTRACT OF PROGRAM PROJECT

SMOKING AND LUNG DEVELOPMENT

Responsible investigator: Paul Hamosh M.D.

CENTRAL THEME: Developmental variations in lung structure affect susceptibility to cigarette smoke in a predictable way.

Project 1. Smoking and Functional lung maturation

Principal investigator: Paul Hamosh M.D., co-investigators: Angelo M. Taveira Da Silva M.D., Max Rabinowitz M.D. and Branislav Vidic S.D.

This project is a composite of four studies: A. The Effect of Smoking on Maximum Expiratory Flow (MEF) in Teenagers. This study is designed to answer the following questions: 1) Do changes in MEF effected by smoking differ according to sex, race and environmental factors (coincident air pollution and economic factors)? 2) Do changes in MEF over the years of development depend on the intrinsic pattern of MEF (in other words, does one pattern of MEF lead to chronic changes upon smoking, while another does not)? The study will be conducted in the Washington Metropolitan area School District in the form of annual lung functions and questionnaires in several schools located in different areas; providing therefore a diverse ethnic, environmental and socio-economic data base.

B. Smoking and "Small Airways" Disease. A Study in Pathology

A Study designed to correlate smoking history, post-mortem lung functions and lung pathology in young victims of sudden death. This study will be in co-operation with the medical examiner. It will test the hypothesis, that those subjects having pathology associated with smoking have different airway patterns than subjects who have no pathology. This study provides the morphological basis for study A.

C. Mechanical Stresses and Lung Pathology

This study will attempt to answer the question: Do mechanical forces contribute significantly to the deformation of airways and lung parenchyma and is the degree of damage related to intrinsic variation in lung structure? This will be a study in animal models, where structural changes will be induced by surgical manipulation or proteolytic enzymes. The animals with altered lungs will be exposed to mechanical lung stress. Morphometry of these lungs, when compared to controls, will provide the answer.

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D. The Effect of Smoking a Single Cigarette on the "Small Airways"

This study is essentially a continuation of the study presently supported by the CTR (#878). It is an attempt to separate susceptible from non-susceptible subjects by "provocation" test, i.e. acute exposure. The question asked is: How is the response affected by ethnic and racial background 2) alteration of smoke (e.g. filters) and 3) pretreatment by pharmacological agents?

These questions are answered by sophisticated plethysmographic studies before, during and after smoking of Kentucky brand cigarettes by volunteers.

PROJECT 2

Smoking and the Biochemical Development of the Lung

Principal investigator: Margit Hamosh Ph.D., Co-investigators: T. Goeringer Ph.D., P. Hamosh M.D., B. Vidic S.D., J. Wrathall, Ph.D.

This project has two main thrusts:

1) To establish the maturation of biochemical pathways in the prenatal and postnatal period and 2) study selected enzymes the same way. The effect of exposure to cigarette smoke on the biochemistry of developing lung will be a major component of this survey. Rodents and primate lungs will be studied at various phases of their development as to their ability to incorporate radio labeled substrates for protein, carbohydrate and lipid synthesis. The biochemical findings will be correlated to functional studies with particular emphasis on lung mechanics in isolated lungs. The biochemical consequences of smoke exposure and structural alteration will also be studied.

PROJECT 3

Smoking and the Cellular and Embryological Aspects of Lung Development

Principal Investigator: Jean R. Wrathall Ph.D.
co-investigators: T. Goeringer Ph.D., M. Hamosh Ph.D. and B. Vidic S.D.

In this project, the effect of smoking and other environmental factors on the growth and development of isolated lung cells will be studied. The embryological approach is twofold: 1) To produce by manipulation of the embryo or the neonate aberrant patterns of airway development and then study the effect of smoke on the adult; and the reverse 2) study the effect of perinatal smoke exposure on development of lung structure (morphometric analysis).

While reading these abstracts might impart some superficial overview of the aims and scope of this proposal, frequent perusal of the diagramms and flowsheet is recommended. A list of recent reviews and editorials dealing with lung development follows:


1. Farrell, P.M. and M.E. Avery. Am. Rev. Resp. Dis. 111: 657, 1975.
2. Thurlbeck, W.M. Am. Rev. Resp. Dis. 111: 803, 1975.
3. Kilburn, K.H. Am. J. Med. 58: 591, 1975.
4. Symposium on Lung Growth and Development. A.T.S. Pediatric Assembly - CTS. Montreal, Canada, May, 1975 (available on tapes only).


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SMOKING AND LUNG DEVELOPMENT OVERVIEW P. HAMOSH M.D.

## General Introduction

The association between cigarette smoking and chronic obstructive lung disease (COLD) has been established by the famous report of the Surgeon General (1) however, to this day, this association has not been proven on a cause effect basis. In recent years, with the discovery of ever increasing numbers of other environmental agents associated with COLD(2) and the increasing realization that host factors might be as important as environmental factors(3) there is an inclination to look at COLD as a multifaceted disease. To this, one should add the realization, that smoking has become a part of our culture and the number of smokers is steadily increasing (4) therefore, one could see only two logical approaches to attempt to dissociate smoking from COLD: 1) to identify the possible harmful substances in cigarette smoke and eliminate them; 2) to identify the subjects among smokers who are at increased risk due to hereditary or acquired factors and dissuade them from smoking.

This later approach is the basis of our working hypothesis. It has been common knowledge that numerous heavy smokers live to very advanced age. Are these people "protected" from the effect of cigarette smoke by some special defense mechanism, or are the ones who develope COLD particularly "susceptible" because of some additional factors which make them vulnerable to cigarette smoke. Since the outset of our interest in the effect of cigarette smoke on the airways, we have been engaged in an intensive search for means to differentiate susceptible individuals from non-susceptible. Our original hypothesis was that there are "REACTORS" and "NONREACTORS" to cigarette smoke. We have extensively studied the effect of smoking a single cigarette on the airway responses (5) we have found that all subjects had acute bronchoconstriction and there was no bimodal distribution to justify division into two groups. Recent studies in black people (6) showed no differences in lung function between smokers and non-smokers. This fact, combined with the well known "resistance" of the black race to COLD forces us to reexamine the question on a racial basis (projects 1A and 1D).

Our original working hypothesis (1970) was based on the postulation of major differences in inervation of the large and small airways (7) this was at the time when the "small airways" (less than 2 mm in diameter) became the center of attention as the possible site of early lung disease (8) While different patterns of response involving large or small airways were shown to exist in hemp workers upon exposure to the dust (9) and differential inervation of the airways demonstrated (10) we are only now showing differences in large and small airways after exposure to cigarette smoke. These changes are not in the magnitude but rather in the time course of the response (11).



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Recent investigations in humans gave us the material which serves as the foundation of our present working hypothesis: first, that it was shown that the resistance of the small airways varies widely in young human lungs (12) and the major determinants of expiratory flow were the number and diameter of airways. The second striking discovery was the reduced number of airway generations in lung from early emphysema (13). Could it be that the geometrical configuration of the airways is the primary determinant of "susceptibility" to environmental insults? Is it possible that the Shape of the conducting system of the lung determines the fate of an individual; whether he will develop COLD or not? Furthermore, if this hypothesis is true, how will one go about identifying the different shapes.

Green et al. (14) studied the maximum expiratory flow-volume (MEFV) curve in a number of healthy young subjects. They found considerable variability and comment on the possibility that one pattern of lung structure and function disposes toward a certain pathological response to a noxious stimulus, while another pattern produces a different pathology or no pathology at all. We know that structural weakness predisposes to destruction, when exposed to mechanical stresses (15), but then certain structures are more susceptible to destruction based on the design of the structure itself (e.g. suspension bridges vs. pillared ones). The complex relationship between structure, function and stresses will be explored in project 1B and 1C.

In addition to the possibility of inherent differences in patterns of lung structure (which might explain the difference in susceptibility to COLD between the white and the black race), one should also consider the environmental factors which might alter the patterns of lung structure during prenatal and postnatal development. Infection during early life is one factor, which might alter or arrest lung maturation and result in abnormal lung pattern. Exposure of the mother to noxious agents might have the same effect on the offspring. These children might grow up as "physiologically" normal subjects, while having a pattern of lung structure which marks them for trouble provided they expose this faulty patterns to environmental stresses.

The following three projects will attempt to 1) identify these patternsmorphologically, 2) to identify the functional expression of these patterns, 3) to produce different patterns in the experimental animal and challenge by environmental insults these animals.



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1. Smoking and Health Report of the Advisory Committee on Smoking and Health to the Surgeon General of the Public Health Service USDHEW, 1964, Public Health Service Publication #1103.

2. Menzel, D. B., Editor. Arch. Int. Med. Symposia 9:10279, 1971.

Br. Med. J. 1:590, 1974.

Lancet 2:1134, 1973.

3. Green, G. M. Am. Rev. Resp. Dis. 102:691-703, 1970.

4. The Health Consequences of Smoking DHEW Publication #(CDC)74-8704

5. Da Silva, A. M. T. and P. Hamosh. J. Appl. Physiol. 34:361, 1973.

6. Seltzer, C. C., A. B. Siegelaub, G. D. Friedman and M. F. Collen. Am. Rev. Resp. Dis. 110:598, 1974.

7. Proposal #878 Council for Tobacco Research. P. I. Paul Hamosh, M.D.

8. Hogg, J. C., P. T. Macklem, and W. M. Thurlbeck. N. Eng. J. Med. 278:1355, 1968.

9. Bouhuys, A. and K. P. van de Woestijne. J. Clin. Invest. 49:106, 1970.

10. Cabezas, G. A., P. D. Graf, and J. A. Nadel. J. Apply. Physiol. 31:651, 1971.

11. Hamosh, P., and A. M. Taveira Da Silva. Unpublished observations.

12. Niewoehner, D. E., J. Kleinerman and D. B. Rice. N. Eng. J. Med. 219:755, 1974.

13. Henderson, R., A. Hislop and L. Reid. Thorax 26:195, 1971.

Hislop, A. and L. Reid Thorax 25:682, 1970.

14. Green, M., J. Mead, and J. M. Turner. J. Apply Physiol. 37:67, 1974.

15. Caldwell, E. J., R. D. Powel and J. P. Mullooly. Am. Rev. Resp. Dis. 102:516, 1970.



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OVERVIEW

Program Project
Smoking and Lung Development

P. HAMOSH M.D.

14. First year budget:

| A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) | % time | Amount |
|---|---|---|
| Paul Hamosh M.D. | 60% | 12,427 |
| A. Taveira Da Silva M.D. | 50% | 14,794 |
| Margit Hamosh Ph.D. | 100% | 26,053 |
| Jean R. Wrathall Ph.D. | 50% | 11,000 |
| Post-Doctoral Fellow | 100% | 14,202 |
| | | 78,476 |
| Technical | | |
| Administrative Assistant | 100% | 11,816 |
| 6 Technicians | 600% | 67,597 |
| Programmer | 20% | 3,906 |
| | | 83,319 |

All Salaries Include 18.35% Fringe Benefits
(16.7% 1/1/76-6/30/76 and 20% 7/1/76-12/31/76)

Sub-Total for A 161,795

B. Consumable supplies (by major categories)

Sub-Total for B 35,000

C. Other expenses (itemize)

Sub-Total for C 25,500

Running Total of A + B + C

D. Permanent equipment (itemize)

Sub-Total for D 53,200

E

Overhead to be negotiated at 15% or higher of A,B & C

Total request 275,495

The budgets are itemized in the individual project budgets.

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First Year Budget — OVERVIEW

Central Service and Facility — P. HAMOSH M.D

3.

13. Budget: (1st year)

| | % time | Amount |
|---|---|---|
| **A. Salaries (Personnel by names)** | | |
| **Professional** | | |
| Dr. Paul Hamosh | 35% | 12,427 |
| **Technical** | | |
| Administrative Assistant | 100% | 11,816 |
| Programmer | 20% | 3,906 |
| Technician | 100% | 11,816 |
| | Sub-Total | 39,965 |
| **B. Consumable Supplies (list by categories)** | | |
| Office supplies, copying etc. | | 4,000 |
| | Sub-Total | 4,000 |
| **C. Other Expenses (itemize)** | | |
| Publication | | 1,000 |
| Travel | | 2,000 |
| Consultation Fees etc. | | 2,000 |
| | Sub-Total | 5,000 |
| **D. Permanent Equipment (itemize)** | | |
| Scintillation Counter (3 channel) | | 18,000 |
| **E. Overhead (15% of A+B+C)** | Sub-total | 18,000 |
| | Total | 66,965 |

Overhead to be negotiated at 15% or higher of A, B, & C

The distribution of efforts by all investigators is presented in Figure 2

10a

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GEORGETOWN UNIVERSITY CENTER FOR INTERDISCIPLINARY STUDIES IN IMMUNOLOGY

Director: Joseph Bellanti M.D. Deputy Director: Sorell Schwartz Ph.D.

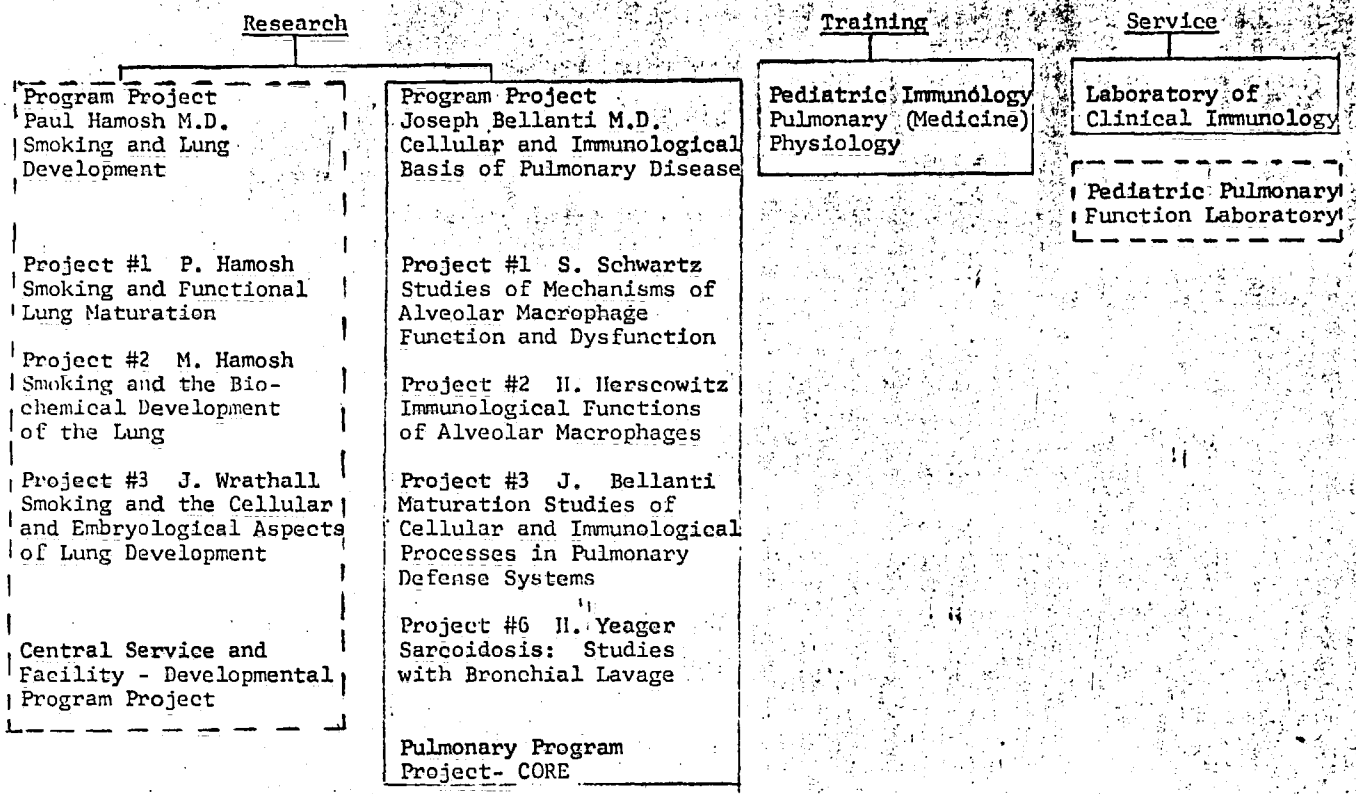


Figure 1.

OVERVIEW

P. HAMOSH M.D.

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## DISTRIBUTION OF EFFORT IN THE PROGRAM PROJECT

### SMOKING AND LUNG DEVELOPMENT

**Central Service and Facility**
Responsible Investigator: P. Hamosh 25%
Administrative Assistant 100%
Computer Programmer 20%
Technician 100%

**Project #1 P. Hamosh 25%**
Smoking and Functional Lung Maturation

A. The effect of teenage smoking on maximum expiratory flow
A. da Silva 30%
Technician 100%

B. Smoking and "Small Airway" Disease: Pathology
N. Rabinowitz 30%
B. Vidic 10%
Technician 100%

C. The Effect Of Mechanical Stresses on Lung Tissue
B. Vidic 10%
Technician 100%

D. The Effect of Smoking A Single Cigarette on The "Small Airways"
A. De Silva 20%
Technician 50%

**Project #2 M. Hamosh 75%**
Smoking and the Biochemical Development of the Lung

Post Doctoral Fellow 100%
J. Goeringer 20%
P. Hamosh 10%
B. Vidic 10%
J. Wrathall 10%
Technician 100%

**Project #3 J. Wrathall 50%**
Smoking and the Cellular and Embryological Aspects of Lung Development

J. Goeringer 25%
M. Hamosh 25%
B. Vidic 10%
Technician 100%

OVERVIEW

P. HAMOSH M.D.

FIGURE 2.

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## DISTRIBUTION OF RESOURCES BY DEPARTMENTS

**Physiology**

P. Hamosh *
A.T. da Silva
Adm. Assist.
technician

Pulmonary Function
Laboratory
Physiology Lab.
Office Headquart.
Computer terminal

Vivarium

Animal Laboratory
(P. Hamosh)

National Biomedical
Research Foundation
Programmer

Computer

**Anatomy**

B. Vidic *
J. Goeringer
M. Hamosh
J. Wrathall
Post-Doc. Fellow.
2 technicians

Microscope Suite
Biochemistry Lab.
Tissue Culture Suite
Embryology Lab.
Radioisotope Lab.

**Pathology**

M. Rabinowitz
technician

Histopathology
Laboratory

**Microbiology**

H. Hezscowitz *

Smoking Machine
Immunology Lab.

**Pharmacology**

S. Schwartz *

**Pediatrics**

J. Bellanti *

**Medicine**

H. Yeager *
(P. Hamosh)

* Members of the center.

Figure 3.

OVERVIEW

P. HAMOSH M.D.

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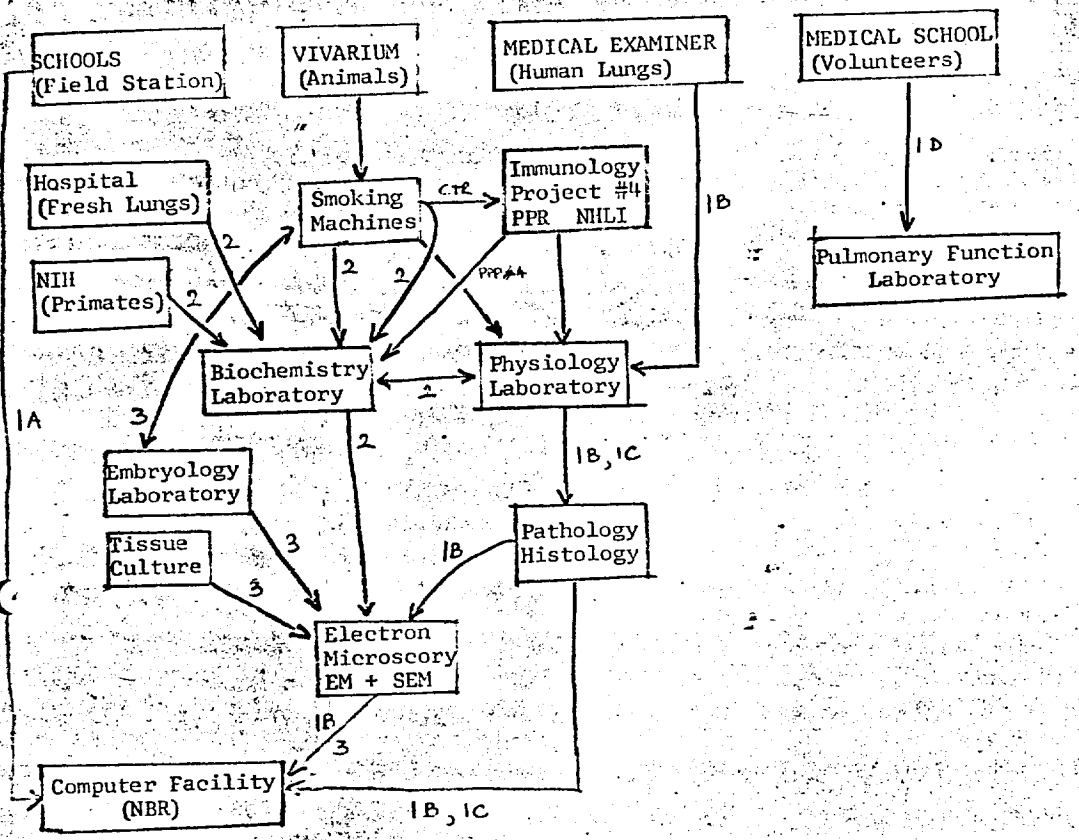
OVERVIEW

INTERACTION OF SOURCES AND LABORATORIES
IN THE DEVELOPMENTAL PROGRAM PROJECT




The Numbers Represent The Projects Described in the Proposal

FIGURE 4.



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[redacted]

PROJECT 1

[redacted]

[redacted]

[redacted]

--PROJECT I

P. HAMOSH M.D.

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: 6/30/75

1. Principal Investigator (give title and degrees):
Paul Hamosh, M.D. Associate Professor of Physiology & Biophysics & Medicine

2. Institution & address:
Georgetown University
37th & O Sts., N.W.
Washington, D.C. 20007

3. Department(s) where research will be done or collaboration provided:
Departments of Physiology, Anatomy, Pathology, the School Systems and the Medical Examiner

4. Short title of study:
SMOKING AND LUNG DEVELOPMENT: A PROGRAM PROJECT.
PROJECT #1. SMOKING AND FUNCTIONAL LUNG MATURATION.

5. Proposed starting date: 1/1/76

6. Estimated time to complete: 5 years

7. Brief description of specific research aims:
This project is a composite of four studies: A. The Effect of Smoking on Maximum Expiratory Flow (MEF) in Teenagers. This study is designed to answer the following questions: 1) Do changes in MEF effected by smoking differ according to sex, race and environmental factors (coincident air pollution and economic factors)? 2) Do changes in MEF over the years of development depend on the intrinsic pattern of MEF ( in other words, does one pattern of MEF lead to chronic changes upon smoking, while another does not)? The study will be conducted in the Washington Metropolitan area school district in the form of annual lung functions and questionnaires in several schools located in different areas; providing therefore a diverse ethnic, environmental and socio-economic data base. B. Smoking and "small airway" Disease A Study in Pathology. A study designed to correlate smoking history, post-mortem lung functions and lung pathology in young victims of sudden death. This study will be in co-operation with the Medical Examiner. It will test the hypothesis, that those subjects having pathology associated with smoking have different airway patterns than subjects who have no pathology. This study provides the morphological basis for study A. C. Mechanical Stresses and Lung Pathology. This study will attempt to answer the question: Do mechanical forces contribute significantly to the deformation of airways and lung parenchyma and is the degree of damage related to intrinsic variation in lung structure? This will be a study in animal models, where structural changes will be induced by surgical manipulation or proteolytic enzymes and then the animals with altered lungs exposed to mechanical lung stress. Morphometry of these lungs, when compared to controls will provide the answer. D. The Effect of Smoking a Single Cigarette on the "small airway". This study is essentially a continuation of the study presently supported by the CTR (#878). It is an attempt to separate susceptible from nonsusceptible subject by "provocation"



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test, i.e. acute exposure. The question asked is: 1) How is the response affected by ethnic and racial background. 2) alteration of smoke (e.g. filters) and 3) pretreatment by pharmacological agents? These questions are answered by sophisticated plethysmographic studies before, during and after smoking of Kentucky brand cigarettes by volunteers.



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Project 1: SMOKING AND FUNCTIONAL LUNG MATURATION

A. The Effect of smoking on Maximum Expiratory Flow (MEF) in Teenagers

BACKGROUND:

Adult smokers can be separated with different degrees of success from non-smokers on the basis of lung function tests, depending on the sophistication of the test and the population studied (1). Most extensive studies were done in white subjects (2). Recent studies indicate that such separation is impossible in blacks and orientals (3). Less is known about lung functions in smoking teenagers.

Cigarette smoking was linked to pathogenesis of heart and lung disease (1). Whereas smoking a cigarette produces immediate changes in airway conductance and heart rate (2), it is less clear what the long term effects are. All smokers develop lung function changes (3), however, it is not clear whether these changes reflect the fact that a person is a smoker, or are only indicators of early lung disease. Many smokers develop chronic lung disease, but the vast majority do not. The question is: are these latter smokers more "protected" or "less susceptible"? No tests are presently available that separate "less susceptible" or "protected" individuals from "susceptible", in spite of major efforts by many groups, including ours.

Young smokers have early functional abnormalities (4), some of which are reversible after discontinuation of smoking (5,6). Sometimes these functional abnormalities are accompanied by respiratory symptoms such as cough, phlegm production, etc. (7). The prevalence of respiratory symptoms in adolescents, (11 years and older) smoking more than 5 cigarettes a day approaches the one reported for adult smokers (7). There is a correlation between smoking habits and respiratory symptoms which might suggest that chronic bronchitis starts early in life (7). Seely et al., (8) have reported significant changes in lung functions in smokers, age 15 to 19 years. They used the maximum expiratory flow volume (MEFV) curve (see figure 1). This test has been shown to be a sensitive detector of early functional changes, both in adults (9,10) and children (11). The incidence of respiratory symptoms increased as cigarette consumption increased. More smokers than non-smokers had MEFV curves convex towards the volume axis. Flows at 50 and 25% of the vital capacity ($MEF_{50}$ and $MEF_{25}$) were significantly lower in those boys and girls smoking more than 15 and 10 cigarettes respectively (8). One out of five teenage smokers had abnormal lung functions not detected by conventional spirometry (12).



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In rapidly growing children, lung size increases faster than total body size (13). Therefore, the "growth" of the MEFV curve is related to total lung capacity. Most normal children have MEFV curves that are concave towards the volume axis . It was suggested that "damage" caused by cigarette smoking at an early age may affect lung development (14). On the other hand, Green et al. (15) suggested that basic morphologic reapportion between lung parenchyma and airways may be a factor in the pathogenesis of disease, preceding exposure to any noxious agents. Certain patterns of lung structure may predispose to lung disease when associated with cigarette smoking. They introduced the word "dysanaptic" for lungs that do not develop isotropically Damage to the lung when still growing could further disturb the airway parenchyma relationship. Recent work by Reid et al. (16,17) suggests that abnormal subdivision of the bronchi might be one of the causes of emphysema, a concept recently amplified by Kilbourne (18). Zapletal et al. (19) suggested that one of the abnormalities of cystic fibrosis is a "maturation arrest" of the lung.

We assume then, as a working hypothesis, that hereditary, congenital or acquired (early in childhood) anatomical changes might produce a respiratory pump which is predisposed, (by virtue of its mechanical properties) to chronic obstructive lung disease (COLD) when associated with other environmental factors, such as smoking, air pollution and infection.

PRINCIPAL AIMS:

1. Study the evolution of the maximum expiratory flow volume curve (MEFV) in children and adolescents.

2. Correlate history of early lung disease and smoking habits with the evolution of the MEFV curve, to identify the type of curve associated with susceptibility to COLD.

3. Analyze these data also, in relation to sex, ethnic background, economic factors, air pollution, etc.

EXPERIMENTAL DESIGN:

To realize the above aims, a prospective study utilizing school age children will be initiated with the following principles in mind:

1. A wide data base, to assure continuity
2. Simple and non-invasive tests, to assure child and parent cooperation
3. Minimum disruption of classrooms, to assure cooperation of school authorities.



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4. A modern data acquisition, storage and retrieval system, to assure rapid processing of information.

MATERIALS AND METHODS:

The subjects will be school children between the ages of 11 to 18. A number of elementary, junior high and high schools will be selected to represent an ethnic and socio-economical cross section of the Metropolitan Washington area. We will aim for 1,300 students annually, considering the demographic realities and frequency of migration (See epidemological and statistical consideration).

MODE OF OPERATION:

The parents will receive a detailed explanation of the purpose and scope of the study signed by the school principal. The parent will return a signed consent form and answer a few simple questions such as: the parent's smoking habits, family and child's history of lung disease, possible exposure to pollutants, etc. The child will then proceed with this consent form to the testing station in the school where he will answer a few questions regarding smoking history. His vital statistics will be taken and spirometry performed. The data will be processed at the processing station at the University and then stored for easy retrieval.

DATA ACQUISITION:

1. Questionnaires (Samples appended).. The design is conducive to simplicity and coding for punch cards.

2. Spirometry Three consecutive maximum expiratory efforts will be performed by the child through a pneumotachograph. Flow will be integrated into volume and the two analog signals (flow and volume) digitized on line and recorded by a digital cassette recorder. The analog signal will also be displayed in real time on a storage oscilloscope for monitoring purposes.

DATA PROCESSING:

A. Theoretically Maximum expiratory flow (MEF) is effort independent below 70% of vital capacity and therefore an ideal, highly repeatable measurement of



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mechanical lung function (20). The magnitude of MEF at any given lung volume is determined by the elastic recoil of the lung ($P_L$) and the resistance of the "upstream" segment of airways ($R_{us}$) called so because they are upstream from the point where airflow limitation occurs (21). Since early changes in COPD are probably in the "small airway" (those less than 2mm in diameter), early changes in MEF at low lung volume would be expected (22). If there are developmental and/or maturational abnormalities or variants in airway anatomy they are more likely to be in the small airways and therefore cause a change in the $R_{us}$ and consequently in MEF at low lung volumes, in other words, the mid or terminal part of MEF is probably the measurement worth the maximum attention when looking for subtle changes in small airway function (See also progress report of Grant # 878).

B. PRACTICAL MEASUREMENTS: (Read with accompanying diagrams)

Maximum expiratory flow will be performed through a disposable mouthpiece connected to a corrugated tube leading into a #3 Fleish pneumotachograph (PNTG). The pressure drop across the PNTG will be picked up by a differential pressure transducer (Validyne PM 46) and amplified by a carrier amplifier. This flow signal will then be integrated by an integrator. (This whole apparatus will be purchased from Hewlett-Packard, who have developed a compact package. However, at the time of writing this proposal, we are evaluating two more systems). The output is two analog signals: flow and volume. These could be recorded by digital or FM analog tape recorder. We are in the process of evaluating both methods to arrive at a simple and inexpensive solution. To minimize cost we are trying to develop a system which can be interphased by existing computational facilities so that the great volume of information could be rapidly processed. During the actual measurements under "field conditions" the flow and volume signals will be displayed on a storage oscilloscope for immediate evaluation of the correctness of procedure and quality of tracing. The signals will be A/D converted or directly taped at the "field station." The record consists of a segment on a magnetic tape and the questionnaires.

C. PROCESSING AT "HOME STATION"

The following derived parameters will be obtained by digital computer:
Observed and Predicted Values for:

1. Forced Vital Capacity (FVC)



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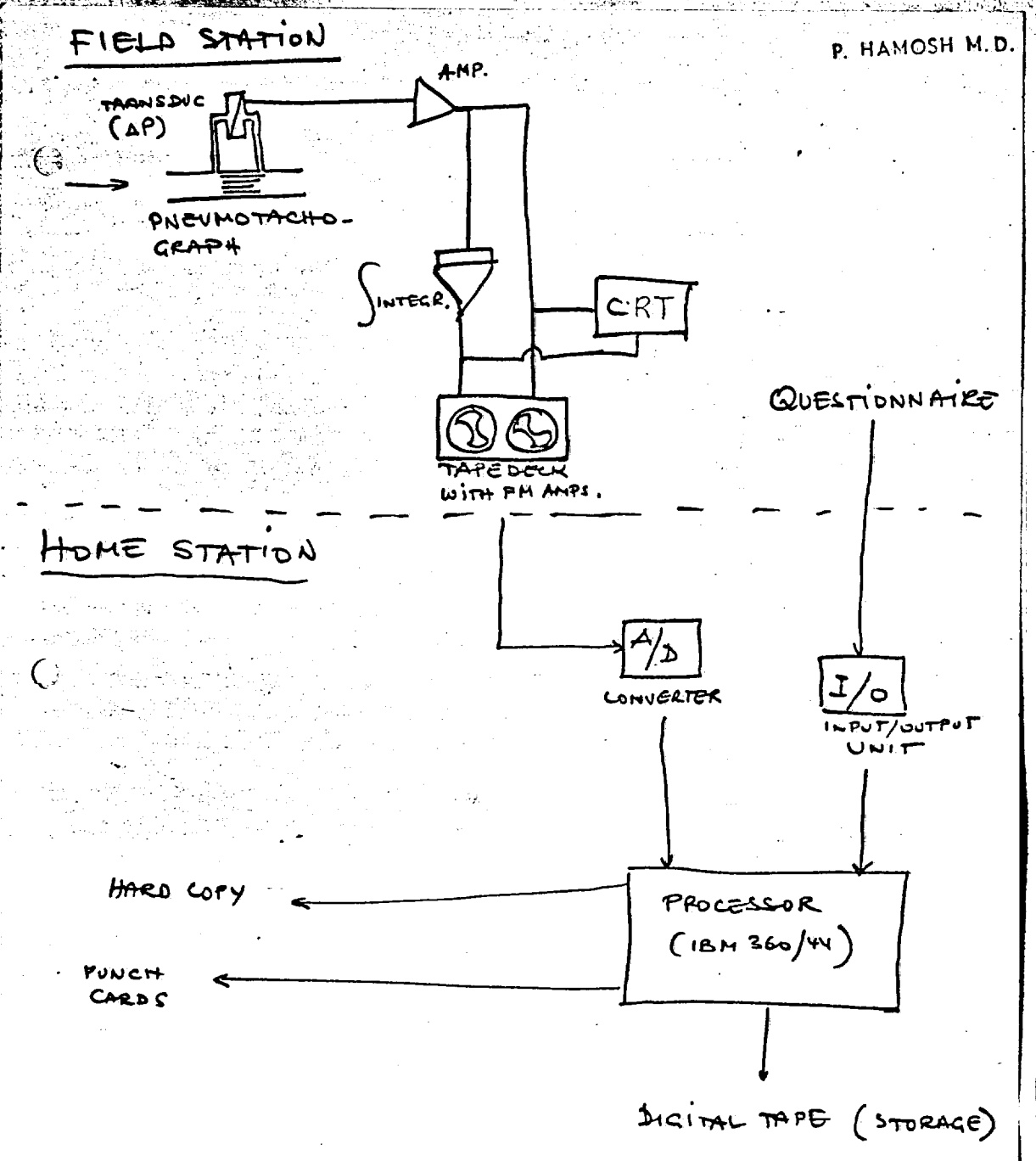


FIGURE 5.



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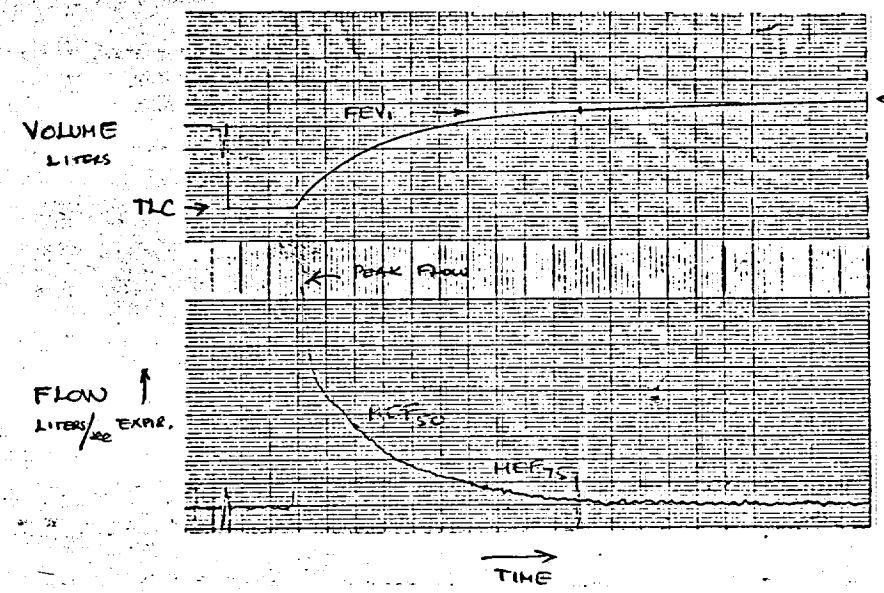
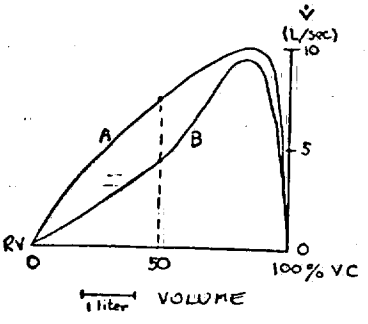
## EXPIRATORY SPIROGRAM




## MAXIMUM EXPIRATORY FLOW-VOLUME CURVE




A REPRESENT THE MEFV CURVE AS SEEN IN YOUNG ADULTS

B IS MORE CHARACTERISTIC OF ADULT SMOKERS

FIGURE 6.

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2. Forced Expiratory Volume (FEV) at any lapse of time
3. $FEV_1$ to FVC ratio
4. Instantaneous Flow at any point of vital capacity ($MEF_x$)
5. Maximum Mid-Expiratory Flow Rate (MMFR)
6. Terminal Flow Rate ($MTF_{50-75}VC$)
7. Peak Flow
8. Momentum Analysis of the Expiratory Curve.

D. EPIDEMIOLOGICAL and STATISTICAL CONSIDERATIONS :

A recent workshop conducted by the National Heart and Lung Institute (NHLI) on the early detection of lung disease deals at length with the experimental design of epidemiological studies of this nature (23). We consider this study primarily a study of structure and function; however, it would be foolhardy to ignore the epidemiologic implications. Therefore we decided to select the number of our subjects and the demographic composition of the group on the basis of valid statistical criteria. Our preliminary estimate is 1,300 subjects, but at the time of writing this proposal, not all the necessary demographic information was available, for the exact calculations. See the separately submitted addendum.

NOTE: At the writing of this proposal, we are seeking to concert our efforts with other groups planning to engage insimilar prospective studies, provided we can agree on standardizing our equipment. This study might become a part of a co-operative effort. If an accord is reached before September, an addendum to this proposal will be forthcoming.



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Source: https://www.industrydocuments.ucsf.edu/docs/xhdm0000

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PROJECT 1B

Smoking and "small airway" disease, a pathological study.

BACKGROUND:

It has been suggested that chronic obstructive lung disease initially affects the airways less than 2mm in diameters, designated as "small airways" (1). Pathological studies in young smokers who died suddenly showed respiratory bronchiolitis (2). Morphometric studies showed higher incidence of emphysema in young smokers (3) but the airways received less attention. Kleinerman et al.(4) did perform studies on postmortem lungs, which showed a lesser evidence of small airway involvement than suggested by Hogg et al.(1). What is missing to this day is a comprehensive study of physiology, morphology and smoking history methodically performed on a sufficient number of post-morten lungs, to yield statistically meaningful data. The question to answer is: what is the nature and distribution of lesions in the lungs of young people, smokers and non-smokers and how does it correlate to life history and ante or post-mortem lung function? The aim of this study will be to accumulate over several years about fifty lungs from young subjects, victims of sudden death, with known history of illnesses and smoking habits; perform pulmonary functions on the isolated lung and then prepare them for histology by pressure fixation. Morphometry will then be performed by automated light microscopy and scanning electron microscopy.

EXPERIMENTAL DESIGN:

The subjects will be selected from the Medical Examiner's cases, preferably subjects who died instantly from head injury, without damage to the chest. If both lungs cannot be obtained at least one whole lung will be used. The subject qualified for study if he has family available for history at a later time, the lung is not leaky and the post-mortem is performed within 24 hours of death.

Pressure volume flow relationship will be studied on the isolated lung (see Methods) which will then be pressure fixed. Subsequently one large sagittal section will be made for mounting on paper. Blocks for morphometry will be selected by random approach (see Methods) and slides prepared for light microscopy and scanning electron microscopy. Morphometry



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will be automated by using pattern recognition computer technology.

METHODS:

1. Lung Mechanics

The isolated lung will be connected by the airway to a pneumotachograph and suspended in a volume plethysmograph. Flow will be measured by the pneumotachograph - differential pressure transducer system, the volume changes by a Krogh spirometer, and transpulmonic pressure by a transducer connected to the airway orifice. All signals will be amplified by carrier amplifiers and displayed on either direct writing recorders or oscilloscopes. The experimental apparatus is shown in Figure 7. A total analysis of volume/pressure (compliance), volume/flow and flow/pressure (conductance) relationships will be measured. Total respiratory resistance and dynamic compliance will be measured also as a function of frequency. For this end, the lung will be oscillated by a high power speaker.

Morphometric Studies:

On completion of the physiological studies, Barium Sulfate dust (Micropaque, Nicholas Laboratories) will be insufflated into the bronchial tree and roentgenograms will be taken at TLC (Total Lung Capacity). The transverse diameter of each segmental bronchus will be measured at a point 0.5 cm from its bifurcation and corrected for magnification. The lungs will be inflated and fixed in 20% neutral buffered formalin in 72 hours at 25 $cmH_2O$ pressure. The lung will be cut into 1 cm parasagittal slices and carefully examined. The amount of emphysema, if present, is determined by point count (6). Ten randomly selected 2 x 2.5 cm sections will be cut from each lobe using a metal template. Transverse sections will be taken from the upper and lower lobe bronchi. The sections will be embedded in paraffin and 6$\mu$m sections will be stained with H & E. Parenchymal sections with significant artifact will be excluded. Six of the remaining sections from each lobe, a total of 60 $cm^2$ fixed tissue, will then be randomly selected for study. The number and size of the non-cartilagenous membranous bronchioles less than 2 mm internal diameter will be evaluated using the method of Matsuba and Thurlbeck (7). First the bronchiole density will be determined and expressed as bronchioles/$cm^2$ fixed tissue. A bronchiole will be counted only if at least 75% of its perimeter clearly consists of membranous bronchiolar wall. This excludes some airways at the edge of sections and some of those cut obliquely



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through the junction of the terminal and respiratory bronchioles. Next, the diameter of each bronchiole (the longest distance in the short plane) will be measured and ranked by size in 50 μm increments. Because the epithelium will be desquamated in some of the lungs, measurement in all cases will be made from basement membrane to basement membrane..

Automated Morphometry

Morphometry of the lung has been greatly improved and standardized in recent years through the work of Weibel, Thurlbeck and Reid (8), but it still represents a very time consuming task. The standard method based on the mathematical work of Tomkieief (9) and Delgado (10) is known as the "point counting" method. A grid of points is superimposed on the microscopic image and the elements coinciding with the point are counted. Needless to say, the number of points is limited and the observer can just count a short time. Because of fatigue, all studies of this nature have to be done double blind, to eliminate bias by the counter.

Scanners have been introduced years age into morphometric work. They have cut the time of counting, however, could not recognize cellular elements of tissue patterns, therefore were limited for use in tasks where no judgement was needed. The next big step ahead was the development of computerized image analysis known as "pattern recognition."

The National Biomedical Research Foundation (NBRF) directed by Dr. Ledley is equipped with a 360/44 IBM computer with all the peripherals to do automated morphometry in this system. The system is described in the appendix. We can count alveoli, bronchi, arteries and other elements. Our own contribution is appended (Ungerleider and Hamosh). This service will be provided to projects 1B, 1C and 3. The personnel of NBRF will also assist in the development of hardware and software for 1A. (See appendix, especially the description of the SPIDAC System).



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Source: https://www.industrydocuments.ucsf.edu/docs/xhdm0000

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Bucher, U. and L. Reid. Thorax 16: 207, 1961 and also 7.

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9. See addendum to follow

10. See addendum to follow



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Source: https://www.industrydocuments.ucsf.edu/docs/xndm0000

P. HAMOSH M.D.

PROJECT 1C

THE EFFECT OF MECHANICAL STRESSES ON LUNG TISSUE.

BACKGROUND:

We have suggested the possibility that shearing stresses in the airways might be an important contributing factor in the pathogenesis of chronic obstructive lung disease or even carcinogenesis (1). Shearing forces generated during maximum expiratory flow (MEF) and particularly during coughing might produce significant damage to bronchial mucosa. (see appended manuscript draft). This damage would be particularly great in the area where sudden narrowing of airways occurs. Such an area exists just downstream from the equal pressure point (EPP). Constant degeneration and regeneration of the bronchial epithelium in this area might lead to metaplasia and ultimately malignant changes. Is it a coincidence that bronchogenic carcinoma arises mostly from the third or fourth division of the bronchi (2), the usual site for the EPP during coughing and MEF from high lung volume? The "yield stress", the level of shearing forces necessary to induce irreversible epithelial changes might be reduced when other factors weaken the epithelium. Such factors could be proteolytic enzyme, local edema or inflammation produced by chemical, immunological or infective agents, etc. Under these conditions, the small airway could also become a site of damaging shearing stresses.

Simple "perpendicular" stresses also become a factor as we approach the terminal lung units. These stresses produce strain on the tensile elements of lung parenchyma and these are well defined and limited (3). If increased mechanical wear and tear is combined with structural weakening, the tissue will yield, i.e.: there will be alveolar septal disruption. Translating this to a human example: given two subjects with the same structural weaknesses in the lung (e.g. alpha$_1$ antitrypsin deficiency) the subject exposed to mechanical stresses of the lung (heavy laborer, smoker with cough) will develop emphysema more rapidly. Our working hypothesis is: mechanical stresses and structural weakness are additive in nature, and both shearing and perpendicular stresses could lead to irreversible lung damage conducive to chronic obstructive lung disease and carcinogenesis.



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The evidence so far is slim, since very little work has been performed in this area. We showed that shearing stress can indeed disrupt isolated tracheal epithelium (1 and appendix). We also made attempts by exposing rats to papain and have half of them swim for two hours a day for one week after papain exposure. The differences in lung structure and function were not significant. We fault the experimental design because the effort of swimming was insufficient. The experimental design we expect to give results is described below:

This hypothesis raises the interesting possibility that cough induced by irritants might be more responsible than the irritant itself for the effects. How does this tie in with lung development? If anisotropic lung growth or dysanapsis (4) on the one hand and developmental arrest, such as suggested after bronchiolitis (5) or cystic fibrosis (6) on the other, lead to the development of smaller airways, the effect of shearing stress will be magnified exponentially. The consequences of perpendicular stresses will occur earlier in a subject who has structural weakness (increased proteolysis, connective tissue displasia, etc.) and may even lead to maturation slowdown, which in turn yields "dysanaptic" lung and the vicious circle is closed.

PRINCIPAL AIMS

The principal aims of this study are:

1. To establish the "yield" stress of lung tissue in vivo to perpendicular and shearing stress.

2. To produce models of decreased "yield" stress by adding proteolysis to stresses.

3. To attempt to correlate sites of maximum "yield" to maximum damage in the animal and human.

EXPERIMENTAL DESIGN - GENERAL

1. The effect of shearing stress

a. Rats and hamsters will be used in these experiments. Tracheal stenosis will be produced in rats by creating a sudden stepwise narrowing by surgical procedure. The animals will be sacrificed at set time intervals and the transitional area examined for damage under light and scanning electron microscopy (see appended manuscript for methodology).



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b. Homotransplants of hamster trachea (7) will be stressed chronically and then inspected for epithelial damage.

c. Human postmortem material will be examined for coincidence of epithelial damage with high shear distribution (see also Project #1B).

2. The effect of perpendicular stress

Assuming that hyperventilation of exercise is the most natural model for increased mechanical lung stress, rats or hamsters will be exposed to a proteolytic enzyme and then divided into a control group and an exercise group, using a treadmill. Alternative models would be total body restraint (8) or the "whirling" mice (9). After a suitable period, the animals will be sacrificed and lung mechanical properties and morphometry compared. The developmental aspect will be explored by using animals at different stages of their growth.

Project 1/B

METHODS:

a. Shearing Effects (see appended manuscript)

The airways exposed to shearing stress will be excised and mounted in longitudinal strips. Part of this strip will be fixed for longitudinal sectioning and staining and other parts will be prepared for scanning electron microscopy. The damage produced by shearing stress will be graded by the method introduced by us (10). Local shearing stresses will be calculated by exposing plastic casts of the stressed airway to in vivo flow rate and measure pressure drops. These methods are described in some detail in the appended manuscript. Results will be interpreted by correlating local shearing stress to epithelial damage.

b. Perpendicular Mechanical Stress (See flowsheet)

The effects of perpendicular stress are best studied by correlating the amount of hyperventilation to damage of lung structure as determined by morphometry.

1. Method of Stressing:

Hamsters will be exercised on a Quinnton rodent treadmill once a day for two hours and four hours respectively. Half of the hamsters will be pretreated by collagenase and elastase administered by nebulization. Stressing will only be done after recovery from initial acute damage. The methodology was described (11).



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2. Measurement of Elastic Properties and Tensile Strength

Upon sacrifice of the animals the lung will be removed in toto and the volume-pressure relationship measured by the static step by step deflation method (12). The tensile strength will be measured by the method of C. J. Martin (13).

3. Evaluation of Damage

Morphometric analysis will be made of the pressure fixed lungs (14). The internal surface area (15) and the pattern of destruction of alveolar septae (16) will be studied (see section on morphometry).

Significance

If shearing or perpendicular stresses do contribute significantly to disruption of airways and alveoli, they probably represent a significant additive factor in the pathogenesis of COLD. It is not now known how cough or hyperventilation affect lung structure. Since clarification of this problem might influence the management of COLD, this is also a clinically important study. The developmental aspect of the problem is in the study of the "yield stress" as a function of age. Is the developing lung more or maybe less susceptible to damage from mechanical stress. While some information exists about the composition of the lung as a function of age (17), very little is known about tensile strength of the lung (18) as a function of age.



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P. HAMOSH M.D.

| Group A | Group B | Group C | Group D |
|---|---|---|---|
| Nonexercised (Care) Controls | Exercised Controls | Exercised Emphysematous | Nonexercised Emphysematous |

↓

Lung Mechanics + Tensile Strength

↓

Pressure Fixation

↓

Embeding, Sectioning, Mounting and Staining

↓

Morphometry for internal surface area (ISA) and patterns of lung destruction

↓

Analysis of Data: Correlation between stress, emphysema, the compliance curve, lung recoil, tensile strength and the magnitude and pattern of lung damage

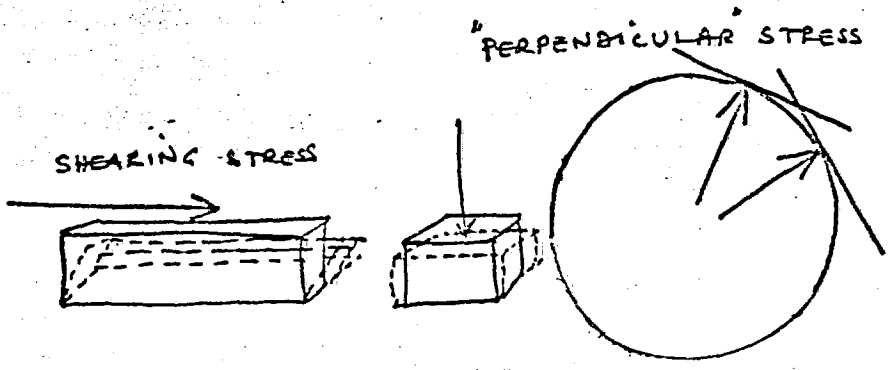


FIGURE 8.



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Source: https://www.industrydocuments.ucsf.edu/docs/xndm0000

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P. HAMOSH M.D.

PROJECT 1/D

The effect of smoking a single cigarette on the "small airways".

BACKGROUND:

This is a continuation of our research on this subject (1). We have now established that the response is immediate (plateaus after three puffs); it is not dose dependent, but it is cigarette dependent (Kentucky brand 1R1 shows a more profound effect than brand 1A1); it probably exerts first effect on the large airways (airway resistance) and the effect on "small airway" (midexpiratory flow) is delayed and also slower to return to normal after cessation of smoking (2).

We still have not resolved whether a "lung mechanics" test will be able to separate "reactors from non-reactors". Presently we are engaged in the study of ventilation distribution as a measure of single cigarette effect. Our results on the effect of a single cigarette were corroborated by Bouhuys et al (3), and are frequently cited in connection with maximum expiratory flow at 50% vital capacity ($MEF_{50}$) as the most valuable single measurement in separating chronic smokers from non-smokers (4).

AIMS:

We would like to pursue this research for several more years to answer the following questions:

1. Response to the same cigarettes we studied (1r1,1a1 and 1a3 smoked through a Cambridge filter.
2. Response to smoke other than cigarettes
3. Response to cigar or pipe smoke
4. Pretreatment with drugs and modification of response.

EXPERIMENTAL DESIGN:

Young volunteers, mostly students and faculty will smoke a cigarette in a body plethysomograph and "small airway" functions will be measured by the methods described in detail in the appended proposals, progress reports and publications (Ref. 1,2,4, Proposal 1A and progress report of CTR grant #878R2).

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Source: https://www.industrydocuments.ucsf.edu/docs/xnbm0000

References:

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Source: https://www.industrydocuments.ucsf.edu/docs/xhdm0000

First Year Budget
Project 1.

P. HAMOSH M.D.



13. Budget: (1st year)

| A. Salaries (Personnel by names) | % time | Amount |
|---|---|---|
| Professional * | | |
| Angelo M. Traveira Da Silva (A) | 50% | 14,794 |
| Technical* Linda Cooper (A) | 100% | 11,816 |
| To Be Hired (Histology) (Bec) | 100% | 11,053 |
| To Be Hired (Machine) (A&D) | 100% | 10,068 |
| * All Salaries Include 18.35% Fringe Benefits (16.7% 1/1/76-6/30/76 and 20% 7/1/76-12/31/76) | Sub-Total | 47,731 |
| B. Consumable Supplies (list by categories) | | |
| Chemicals, Disposables | | 3,000 |
| Histology, Microscopy | | 2,500 |
| Photographic, Recording | | 2,000 |
| Animals and Care | | 2,500 |
| | Sub-Total | 10,000 |
| C. Other Expenses (itemize) | | |
| Computer Time (A,B) | 100 hours | 9,000 |
| Travel | | 2,000 |
| | Sub-Total | 11,000 |
| D. Permanent Equipment (itemize) | | |
| Computer System "Field Station" And Interface (A) | | 15,000 |
| Custom Made System For Pressure Fixation (B) | | 1,500 |
| Surgical Equipment (B&C) | | 900 |
| Custom Made Plethysmographs (B&C) | | 2,000 |
| | Sub-Total | 19,400 |
| E. Overhead (15% of A+B+C) | Total | 88.131 |

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Overhead to be negotiated at 15% or higher of A,B, & C

The Distribution of efforts by all investigators is presented in Figure 2



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PROJECT 2

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PROJECT 2

P. HAMOSH M.D.

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.
110 EAST 50TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: 6/30/75

1. Principal Investigator (give title and degrees):
Margit Hamosh, Ph.D. Assistant Professor of Anatomy

2. Institution & address:
Georgetown University
37th & O Sts., N.W.
Washington, D.C. 20007

3. Department(s) where research will be done or collaboration provided:
Anatomy, Physiology and Microbiology

4. Short title of study:
SMOKING AND LUNG DEVELOPMENT: A PROGRAM PROJECT
PROJECT # 2, SMOKING AND THE BIOCHEMICAL DEVELOPMENT OF THE LUNG

5. Proposed starting date: 1/1/76

6. Estimated time to complete: 5 years

7. Brief description of specific research aims:
SMOKING AND THE BIOCHEMICAL DEVELOPMENT OF THE LUNG. This project has two main thrusts: 1) to establish the maturation of biochemical pathways in the prenatal and postnatal period and 2) study selected enzymes the same way. The effect of exposure to cigarette smoke on the biochemistry of developing lung will be a major component of this study. Rodent and primate lungs will be studied at various phases of their development as to their ability to incorporate radio-labeled substrates for protein, carbohydrate and lipid synthesis. The biochemical findings will be correlated to functional studies with particular emphasis on lung mechanics in isolated lungs. The biochemical consequences of smoke exposure and structural alteration will also be studied.



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Source: https://www.industrydocuments.ucsf.edu/docs/xhdm0000

## Smoking and the biochemical development of the lung

Background The association between smoking and lung disease was always based on the assumption that the irritant effect of the smoke produces mechanical or chemical changes in the bronchial epithelium which ultimately leads to either narrowing or disruption (chronic bronchitis and emphysema) on the one hand, or metaplastic, oncogenic changes which ultimately result in bronchogenic carcinoma. Most of this association is purely statistical and the mechanism of cause-effect relationship has not yet been established satisfactorily. Since all smokers are equally exposed to the irritating effect of smoke, it is likely that host factors or some adjuvants play an equally important part in the pathogenesis of the disease entities associated with smoking. Indeed, a number of recent observations (1,2,a,b) indicate that hereditary ($alpha_1$ anti-trypsin deficiency), acquired (childhood bronchiolitis) and environmental (air pollution) factors are major determinants in the severity of lung disease developing in association with smoking. One would therefore speculate that the development of chronic lung disease is multifaceted with smoking representing one facet which might become deadly when associated with the other facets as well.

Virtually nothing is known about the influence of cigarette smoke on the developing lung. As a matter of fact, very little is known about the influence of almost anything on the developing lung. Recent work by Lynn Reid (3), Green and Mead (4) and others (5), suggests the intriguing possibility that developmental or maturation arrest, or nonisotropic development of the lung might be responsible for predisposing the lung to the noxious effects of environmental irritants; however, it is equally tempting to specualte that: a) the environmental factors are responsible for the developmental defects, or b) the developmental defects are due to inherent or acquired biochemical disturbances. Therefore, the central theme of this project is the determination of the basic biochemical processes during the development of the respiratory system and the effect of cigarette smoke on the course of this development. Recent work has shown that smoking by the mother exerts a retarding influence on fetal growth (6, a) and leads to a greater number of unsuccessful pregnancies due to either still birth or neonatal death (7), and that "passive" smoking by the child might increase the incidence of respiratory infections (8). If these observations are correct there are two possible ways in which smoking could affect the host: a) it could change the "substrate" for environmental invasion, b) it could weaken the defenses, such as the immune apparatus, or mucociliary clearance. We would like to explore the first possibility and see whether exposure to smoke of developing animals will change their biochemical processes and maturation. The embryonal and cellular responses are the subject of Project 3.



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Specific Background It is increasingly clear that the lung, in addition to its primary role in gas exchange, is a metabolically active organ. Studies during the past few years have shown that the lung consumes oxygen (9,10), utilizes energy yielding metabolites (11,12), and is active in the synthesis of metabolites needed to maintain its structural and functional integrity (12,13). There is a general lack of standardization of the methodology employed in the study of lung metabolism, (see review ref. 13) and as a result we have only a sketchy picture of the biochemical ability of the lung. The only lung component that has been studied thoroughly is the surfactant complex (14,15,16). Indeed, most of the biochemical research in recent years has concentrated on the study of the composition structure and biosynthesis of surfactant (see reviews, ref. 14,15,16,17).

If our knowledge of lung biochemistry is spotty at best in the adult of a number of species, it is almost nil when we approach the perinatal and early developmental period. Here again, the overwhelming importance of the surfactant system in early post natal life, led to in depth investigation of the synthetic pathways of dipalmitoyllecithin (the chief component of surfactant) (16,18,19). There is at present a considerable discrepancy between our knowledge of the anatomical development of the lung and that of its biochemical development; the first has advanced considerably during the last few years, as a result of the elegant studies of Reid (20), Boyden (21), Weibel (22), and their collaborators.

Lung maturation is generally determined by the ability of the pre and immediately post natal lung to synthesize and maintain an adequate concentration of alveolar surfactant (19,23,25). It has been established that the fetal lung reaches this stage a few days to a few weeks before birth, depending on the length of the gestation periods of the species (16,19,23-25).

Lung metabolism (with the exception of surfactant (16,19) and collagen synthesis (26) has been studied chiefly in adult animals. Glucose metabolism in the lung proceeds via glycolysis, Krebs cycle and pentose phosphate pathway (10,26); the lung produces relatively large amounts of lactate even when the $PO_2$ in the tissue is high (10,27). The pentose phosphate pathway which is a source of NADPH is especially important in protecting the lung from oxygen toxicity (28). Energy generating and utilizing pathways in lung tissue have only recently come under investigation (29). We have very limited information on protein synthesis in the adult rat lung (13). We are only starting to obtain information about the detoxifying ability of the lung. The lung has a special role in this respect since it is exposed both through the airways and through the vascular bed to a large number of potentially toxic substances. The role of mucociliary clearance (30) and of alveolar macrophages (31) in the protection of the lung are under active investigation. The role of the capillary endothelial cell has been largely overlooked and is emerging only recently as an active metabolic unit with special enzymatic functions in the uptake of circulating metabolites (32) and in the handling of bioactive substances (33,34).

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This research project sets several aims:

1. To evaluate the major metabolic pathways in the lung during pre-and post-natal development in the experimental animal (rats, rabbits, guinea pigs, mice, primates).

2. To study individual enzymes of special importance to intermediary metabolism such as:

   a. Lipoprotein Lipase, the enzyme that catalyzes and regulates the uptake of circulating triglyceride-fatty acids by the lung (32)
   b. Glucose-6-phosphate dehydrogenase, a key enzyme of the pentose phosphate pathway which maintains a steady supply of NADPH.

3. To study the effect of exposure to digarette smoke prenatally (i.e.: exposing the mother) or postnatally on the biochemical activity of the developing lung. In addition to the evaluation of the major metabolic pathways described in sections 1 and 2 we will measure enzyme activities or tissue components that could affect resistance or susceptibility to the ingredients of cigarette smoke.

4. To study the metabolic pathways of human lung - adult and newborn and correlate the findings to the smoking history of the subjects (adults) or the mothers (newborns).

EXPERIMENTAL DESIGN: The following parameters will be studied using as experimental preparation fresh lung slices: (We have chosen the tissue slice method since it is the least time consuming and according to recent studies (13,27) has an identical glucose metabolism as that of the isolated perfused lung.)

MAJOR METABOLIC PATHWAYS

1. Glucose metabolism. Uptake and utilization of glucose will be studied by measuring the amount of $^{14}C$ labelled glucose metabolized to $^{14}CO_2$ and lactate, and incorporated into tissue lipids.

The relative amount of glucose metabolized through glycolysis and Krebs cycle or through the pentose phosphate pathway will be measured by the use of 1 $^{14}C$ and 6 $^{14}C$ glucose substrate. The ratio of lactate to pyruvate production will be taken as a measure of the cytoplasmic redox state of the tissue; in addition the concentration of ATP and especially the ATP/ADP ratio will be measured.

2. Protein synthesis will be measured by the incorporation of labeled amino acids (leucine) into lung protein.



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3. Glucoprotein synthesis will be measured by the incorporation of labeled glucosamine into lung and tracheal glucoproteins.

4. Lipid metabolism Biosynthesis of lipids (neutral lipids and phospholipids) will be measured by the uptake and incorporation of labeled fatty acids and choline into tissue lipids.

If any changes will be found in the above described pathways during lung development, we will study the individual enzymes with the aim of localizing the specific change.

2. INDIVIDUAL ENZYMES OF SPECIAL IMPORTANCE TO INTERMEDIARY METABOLISM

a. Lipoprotein Lipase

During the transition between intrauterine to post natal life, there is in general a switch from the use of glucose as the main energy source to the use of other substrates, chiefly fatty acids. The ability of a tissue to utilize circulating fatty acids (which are present in the blood as triglyceride-fatty acids in chylomicrons and very low density lipoproteins) is directly related to the activity of lipoprotein lipase, an endothelial enzyme that catalyzes and regulates the uptake of blood triglyceride-fatty acids. We have recently described the enzyme in adult lung (32). The ability of the neonatal lung to utilize circulating triglyceride-fatty acids will be measured as a function of lipoprotein lipase activity.

b. Glucose-6-phosphate Dehydrogenase (G-6-P D), a key enzyme of the pentose phosphate pathway will be measured; this pathway is especially important in maintaining a steady supply of NADPH. An increase in G-6-P D activity has recently been linked to the development of tolerance to oxygen exposure in rats (28)

3. THE EFFECT OF EXPOSURE TO CIGARETTE SMOKE PRE-AND POSTNATALLY ON THE BIOCHEMICAL ACTIVITY OF THE DEVELOPING LUNG

a. Aryl hydrocarbon hydroxylase (AHH) a microsomal mixed function oxidase that metabolizes benz(a) pyrene and other polynuclear aromatic hydrocarbons present in tobacco smoke (35-37). It is of special interest to follow possible changes in the enzyme activity in lungs of fetuses whose mothers were exposed to cigarette smoke.

b. The content of cytochrome P-450 in lung.

c. The effect of antioxidants, such as Vitamin C on the prevention of changes caused by exposure to cigarette smoke.



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4. EFFECT OF CIGARETTE SMOKE ON THE METABOLIC PATHWAYS OF HUMAN LUNG (ADULT AND NEWBORN)

The Experimental Design will be similar to that of Sections 1,2 and 3.

Human Lung Specimens of adult human lung will be obtained during surgery. During the 5 year period of this research project, we hope to accumulate a large enough sample of lungs from smokers and non-smokers.

Newborn lung will be obtained at autopsy. We have the full cooperation of the Department of Neonatology, Georgetown University Hospital, and are confident that the specimens will be obtained immediately after death. Details of the smoking habits of the mothers will be obtained.

MATERIALS AND METHODS

Animals Rats, mice, guinea pigs and rabbits will be sacrificed at several time intervals from the beginning of the last trimester of pregnancy until parturition. The uteri will be removed and placed in cold Krebs Ringer bicarbonate solution; the fetuses will be decapitated and the lungs will be removed and stored in cold buffer until all the specimens have been collected.

Fresh fetal primate lung will be a gift from the NIH primate colony through the courtesy of Dr. Phillip M. Farrell, Chief, Section on Developmental Biology and Clinical Nutrition, Neonatal and Pediatric Medicine Branch, NICHD.

Analysis of tissue components The possible changes in lung composition during maturation and after exposure to cigarette smoke in experimental animals and man will be studied by measuring the concentrations of the following components: protein, DNA, RNA, lipid (total lipid, neutral glycerides and phospholipids).

Exposure to cigarette smoke

1. Pregnant animals

a. Pregnant animals will be exposed to smoke in the Walton Horizontal Exposure smoking machine developed under CTR contract by Process and Instruments Corporation, Brooklyn, New York. The animals will be divided into 4 groups. One group will be exposed to cigarette smoke (1 or 2 Kentucky reference cigarettes IAI or IRI daily) for the whole length of pregnancy; the second group will be exposed during the first trimester only; the third group during the last trimester of pregnancy only; the fourth group will be "chronic" smokers exposed for variable periods of time before pregnancy



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and throughout pregnancy. The animals will be allowed to deliver spontaneously; the composition and metabolic activity of the lung will be studied in the offspring of the four groups. The newborn animals will be divided into several groups and studied from birth until weaning (age 3 weeks). In addition to lung metabolism we will record size, weight and weight gain in the offspring of smoking mothers and compare it to non-smoking controls. For some experiments the pregnant rats will be sacrificed during the last trimester of pregnancy as described above.

Newborn and young animals. These animals will be exposed to two concentrations of cigarette smoke, 1. the equivalent if 1/2 of a IAI Kentucky cigarette daily for 1-2 weeks, and 2. a very low level of cigarette smoke to mimic the "passive" smoking of children.

"passive" smoking is a term to describe the author doesn't seem to understand their

LUNG BIOCHEMISTRY

Tissue Preparation. 1 mm thick lung slices will be prepared with the Mc Ilwain tissue chopper (Brinkman Instrument Co.). It has recently been shown that this is the optimal thickness for the study of metabolism in lung slices (9).

1. GLUCOSE METABOLISM Lung slices (50-100 mg) will be incubated in 2 ml Krebs Ringer bicarbonate (KRB) buffer containing 1uCi U $^{14}$C-glucose for 15-180 min; incubation will be at 37°C in a Dubnoff bath. Each incubation flask will be equipped with a center well for the collection of $^{14}CO_2$ produced during the incubation and will be aerated for 1 minute with a mixture of 95% $O_2$-5% $CO_2$ at the start of the incubation. The reaction will be stopped by the addition of 0.2 ml 30% perchloric acid; 0.2 ml phenethylamine will be injected into the fluted filter paper inside the central well and the bottles will be allowed to stand for 30-60 minutes for the complete release of $^{14}CO_2$ from the medium; the flasks will be opened, the phenethylamine soaked filter paper will be transferred to counting vials containing 10 ml Toluene Scintillator for the quantitation of the $^{14}CO_2$ formed. The tissue will be thoroughly rinsed and homogenized in chloroform:methanol-2:1 for analysis. The incubation medium will be frozen for the subsequent determination of lactate (38). The relative contribution of glycolysis and Krebs cycle vs. pentose phosphate pathway to glucose metabolism during lung development will be studied in experiments in which the tissue will be incubated separately with 1$^{14}$C glucose and 6$^{14}$C glucose and the production of $^{14}CO_2$ with the two substrates will be measured. The $CO_2$ production will be expressed as specific yield as suggested by Katz and Wood (39).

2. PROTEIN SYNTHESIS Lung slices (50-100 mg) will be incubated in 2 ml KRB buffer containing 2uCi of $^{3}$H leucine. The incubation flasks will be aerated throughout the incubation (15-180 mins) with a mixture of 95% $O_2$-5% $CO_2$.



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At the end of the incubation the tissue will be rinsed and homogenized in 5 ml 10% TCA (Trichloracetic acid). The TCA precipitate will be washed x2 with 10% TCA and lipids will be extracted with a mixture of ether: acetone: chloroform- 2: 2: 1 at 50°C for 30 min. The incorporation of labeled amino acids into TCA insoluble lung protein will be quantitated by liquid scintillation spectometry and expressed as dpm /mg protein.

3. GLUCOPROTEIN SYNTHESIS Lung slices and slices of trachea will be incubated in 2 ml KRB buffer containing 2-4 4uCi $^{3}H$ glucosamine. Incubation will be identical to that for the incorporation of amino acids. At the end of the incubation, the tissue will be homogenized in 10% TCA + 1% Phosphotungstic acid to insure quantitative precipitation of glucoproteins. The glucoprotein precipitate will be washed X2 with 10% TCA + 1% phosphotungstic acid and processed further as described under "protein synthesis".

4. LIPID METABOLISM Uptake and esterification of $^{3}H$ palmitic acid will be measured also with the tissue slice preparation. $^{3}H$ palmitic acid will be bound to 4% albumin solution as described by Darrah and Hedley-Whyte (40). 50-100 mg lung slices will be incubated for 15-180 min in 2 ml KRB buffer (slightly modified to contain less $Ca^{++}$) containing 2% albumin and 0.1-0.2 uCi $^{3}H$ Palmitate. The following paramenters will be studied: esterification of palmitic acid, synthesis of total lipids, (neutral glycerides and phospholipids). In some experiments we will measure the amount of palmitate that is used for energy production, i.e: oxidized to $CO_2$, in these experiments the substrate will be $1^{14}C$ palmitic acid. The ability of the lung to synthesize phospholipids, especially lecithin, will be studied also in assay systems containing $^{3}H$ choline as substrate. At the end of the incubation, the tissues will be rinsed in 1% albumin solution (to remove adsorbed fatty acid) and homogenized in chloroform: methanol - 2: 1 for the analysis of lipids.

SPECIFIC ENZYMES OF SPECIAL IMPORTANCE TO INTERMEDIARY METABOLISM

Lipoprotein Lipase activity will be determined in lungs from fetal and newborn rats as described previously (32) (see Appendix) The activity will be measured in acetone-either preparations of the lungs using as substrate doubly-labeled ($^{3}H$-glycerol, $^{14}C$-fatty aid) chylomicron triglyceride (41). Lipoprotein lipase activity will also be measured in isolated, cultured lung endothelial cells obtained in Project 3. Glucose-6-phosphate dehydrogenase activity will be measured by the method of Lohr and Waller (42).



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ENZYME ACTIVITIES AND TISSUE COMPONENTS ACTIVE IN THE METABOLISM OF CIGARETTE SMOKE INGREDIENTS

a. Aryl Hydrocarbon Hydroxylase will be measured in lung preparations by a modification (37) of the method of Nebert and Gelboin (43).

b Cytochrome P450. Methods for the quantitation of cytochrome P450 in the lung are now being developed (44). The methods are extremely elaborate and time consuming for lung tissue; we hope that in 1-2 years, at the time when we intend to embark on this specific question better methods will be available for the measurement of cytochrome P450 in the lung.

c Vitamin C will be supplemented in the diet of pregnant animals exposed to cigarette smoke; we will follow the offspring to see whether this antioxidant has a beneficial effect on the growth and lung metabolism of the newborn. (The Vit C level was found to be significantly lower in the serum of women who smoked cigarettes during pregnancy (45)).

Light and Electron Microscopy (scanning and transmission) will be performed throughout this study to follow:

1. Morphological changes during lung maturation

2. Morphological changes caused by exposure of the animals to cigarette smoke

ANALYTICAL METHODS

Analysis of tissue components The following components of the lung will be analyzed in all experimental specimens (fetal and neonatal animals; after exposure to cigarette smoke and in all samples of human tissue):

1. Protein will be measured by the method of Lowry et al (46).

2. DNA will be extracted and measured by the method of Schneider (47) or for very small tissue samples as described by Burton (48).

3. RNA will be extracted and measured according to Schneider (47) or will be calculated by subtraction of the DNA from the total nucleic acids as described by Laros et al. for human lung (49).

4. Total lipids in all tissues will be extracted and purified according to the method of Folch et al. (50).



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5. Individual lipids in the major lipid classes (neutral glycerides and phospholipids) will be separated by thin layer (40,51) and column chromotography (40,52).

6. Total lipids will be quantitated by the method of Rapport and Alonzo (53).

7. Phospholipids will be measured by the method of Bartlett (54).

Analytical methods used in metabolic studies

1. Glucose concentration will be measured with the glucose oxidase kit (Worthington).

2. Lactate concentration will be measured with the lactic dehydrogenase assay described by Hohorst (38).

3. ATP and ADP will be measured enzymatically (55).

4. Free fatty acids will be separated from esterified fatty acids by the method of Borgstrom (56).

All the methods for glucose metabolism, protein, glucoprotein, lipid and phospholipid synthesis are used at present in our laboratory. All the methods have been adapted to the study of lung metabolism during the last year and are presently used in our study on the effect of cigarette smoke on the metabolism of adult rat lung (CTR, Project 936).

In Project 2 we plan to share smoke exposed animals with Dr. Herscowitz (Department of Microbiology) who will use large numbers of mice in his project.

The methodology developed under project CTR 936, will also be applied to the biochemical studies of project 3. We will study the individual lung cell types cultured in project 3. (Type II alveolar cells, endothelial cells) for the metabolic changes induced by exposure to cigarette smoke. This will enable a comparison between the effect of cigarette smoke at organ level to that at the cellular level.

TENTATIVE SCHEDULE

1. The estimated time period for completion of part 1 (major metabolic pathways in the lung during pre and postnatal development is 12-18 months (1st and 2nd year of project).



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2. The study of specific enzymes, such as lipoprotein lipase, glucose-6-phosphate dehydrogenase (and other enzymes which will be studied as an extension of part 1) will proceed in the 2nd and 3rd year of the project. We hope that at that time cultures of the various types of lung cells will be available from project 3 and that we will be able to study the enzymes in whole lung preparations as well as in preparations of the individual cell types.

3. The effect of cigarette smoke on lung metabolism during the developmental period will be initiated as soon as we have sufficient information about the basic metabolic pathways of the control developing lung. We plan to start these smoke exposure experiments during the 2nd year of the project and to continue throughout the 5 year period.

4. Aryl hydrocarbon hydroxylase (AHH) activity, cytochrome P450 and the effect of antioxidants (Vit C) in lung of animals exposed to cigarette smoke will be studied in the 3rd -5th year of the project.

5 Human lung will be collected and studied throughout the 5 year period of the project in order to obtain a large enough sample of lungs from smokers and non-smokers (adults) or from the children of smoking and non-smoking mothers.



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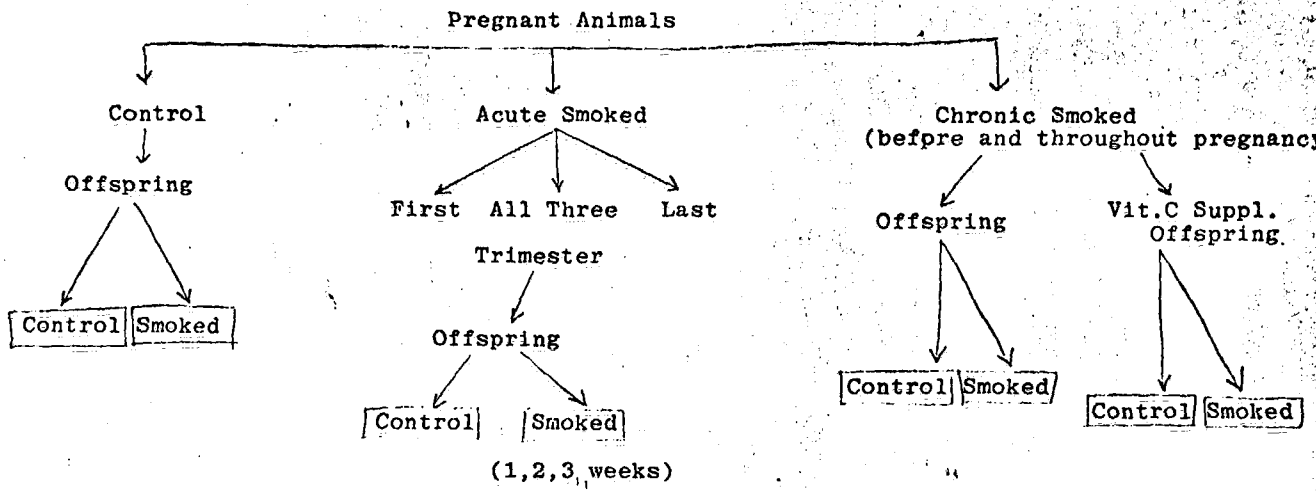
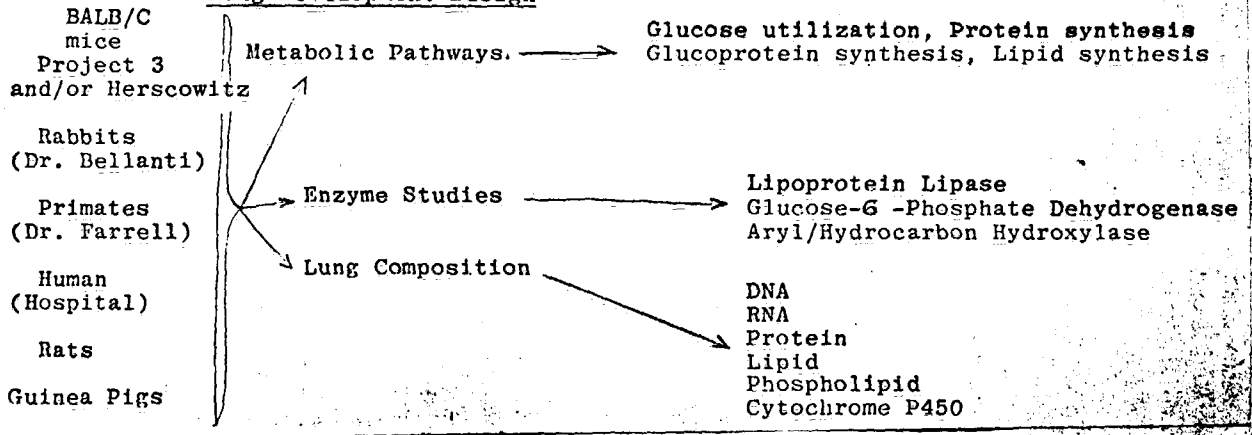
## PROJECT 2 (and 3)

### Smoke Exposure Design




### Lung Development Design




P. HAMOSH M.D.

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First Year Budgets
Project 2.

P. HAMOSH M.D.



13. Budget: (1st year)

| A. Salaries (Personnel by names) | % time | Amount |
|---|---|---|
| Professional | | |
| Dr. Margit Hamosh | 100% | 26,053 |
| Post-Doctoral Fellow | 100% | 14,202 |
| Technical | | |
| Technician | 100% | 11,422 |
| All Salaries Include 18.35% Fringe Benefits (16.7% 1/1/76-6/30/76 and 20% 7/1/76-12/31/76) | Sub-Total | 51.677 |
| B. Consumable Supplies (list by categories) | | |
| Glassware and Disposables | | 4,000 |
| Isotopes | | 2,000 |
| Chemicals | | 3,000 |
| Animals | | 2,000 |
| | Sub-Total | 11,000 |
| C. Other Expenses (itemize) | | |
| Travel | | 1,500 |
| Repair and Maintanance | | 2,000 |
| | Sub-Total | 3,500 |
| D. Permanent Equipment (itemize) | | |
| Centrifuge (GLC) | | 1,500 |
| E. Overhead (15% of A+B+C) | | 67,677 |
| | Total | |

Overhead to be negotiated at 15% or higher of A, B & C

The distribution of effort by all investigators is presented in Figure 2.



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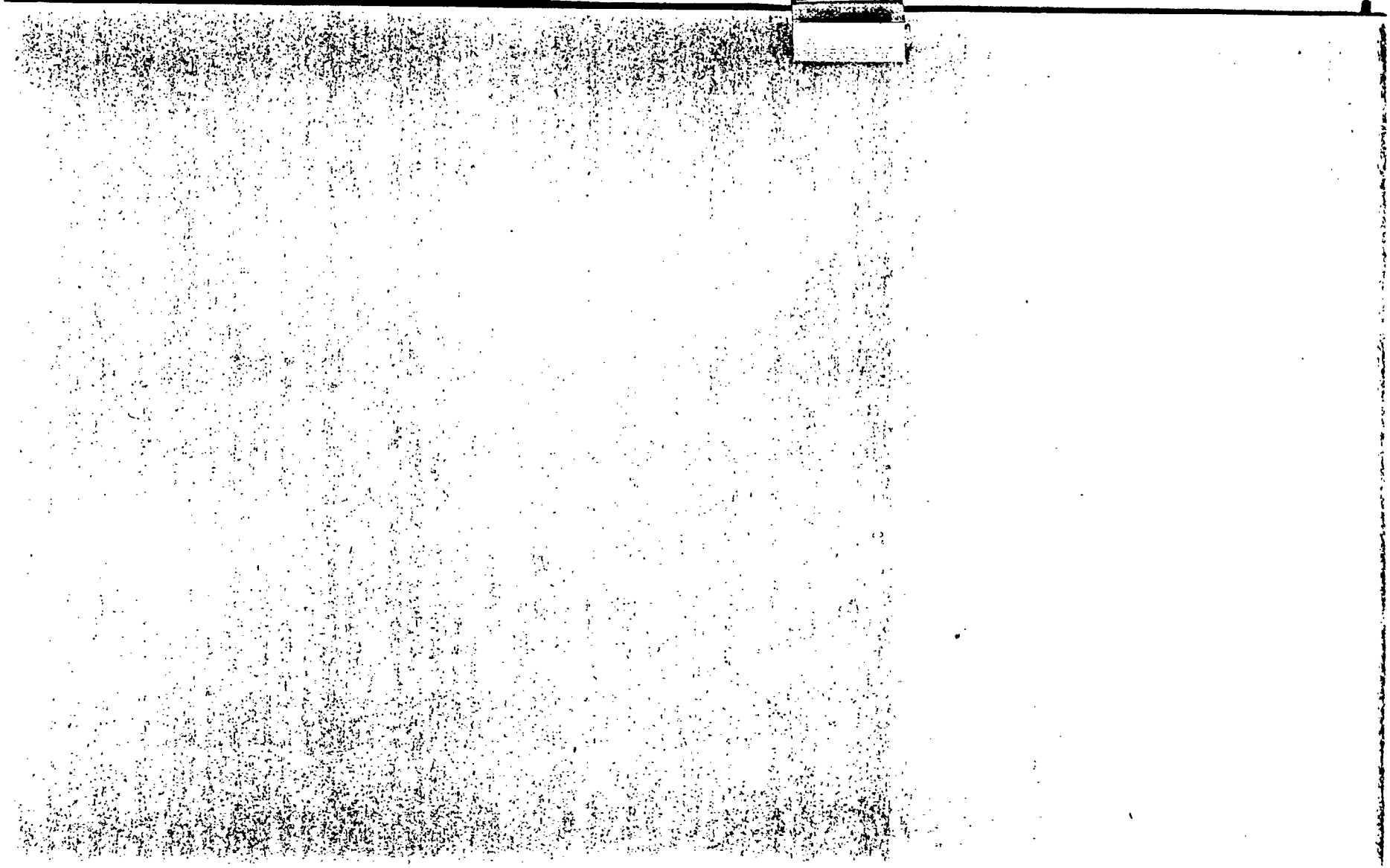
PROJECT 3



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PROJECT 3

P. HAMOSH M.D.

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8855

Application for Research Grant
(Use extra pages as needed)

Date: 6/30/75

1. Principal Investigator (give title and degrees):
Jean Wrathal, Ph.D. Assistant Professor of Anatomy

2. Institution & address:
Georgetown University
37th & O Sts., N.W.
Washington, D.C. 20007

3. Department(s) where research will be done or collaboration provided:
Anatomy and Physiology

4. Short title of study:
SMOKING AND LUNG DEVELOPMENT: A PROGRAM PROJECT.
PROJECT #3, SMOKING AND THE CELLULAR AND EMBRYOLOGICAL ASPECT OF LUNG DEVELOPMENT

5. Proposed starting date: 1/1/76

6. Estimated time to complete: 5 years

7. Brief description of specific research aims:

SMOKING AND CELLULAR AND EMBRYOLOGICAL ASPECTS OF LUNG DEVELOPMENT.

In this project, the effect of smoking and other environmental factors on the growth and development of isolated lung cells will be studied. The embryological approach is two fold: 1) to produce by manipulation of the embryo and the neonate aberrant patterns of airway development and then study the effect of smoke on the adult; and the reverse 2) to study the effect of perinatal smoke exposure on devlopment of lung structure (morphometric analysis).

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PROJECT 3

SMOKING AND THE CELLULAR, AND EMBROYOLOGICAL ASPECTS OF LUNG DEVELOPMENT

General Background:

In project 3 we propose a broad exploration of possible effects of cigarette smoke on: 1) different cell types in the lung; 2) lungs with different morphometric characteristics and 3) on lungs at different stages of development.

Pulmonary emphysema is characterized by destruction of alveolar walls consisting of epithelial, endothelial and interstitial cells. An increased incidence of emphysema has been found in cigarette smokers (1). Yet at present there is no information on the direct effect (if any) of cigarette smoke on the individual cell types comprising the alveolar wall. One of these, the alveolar type II epithelial cell (granular pneumocyte) appears to be intimately involved with the synthesis of surfactant (2,3,4), the substance that prevents collapse of the lung at low air volumes. Evidence suggests that type II cells also give rise to the type I epithelial lining cells (5,6). A second cell type, the alveolar interstitial cells, are the source of collagen and elastic fibers (7) that contribute to the overall elastic properties of the lung. In addition, the interstitial cells play a major role in post-natal alveolar development (8,9,10). Besides mediating transport to and from the blood, the pulmonary endothelial cells actively metabolize a number of vasoactive substances (11) that affect the organism as a whole. Lipoprotein lipase, the enzyme required for processing of plasma cylomicrons is present at or near the plasma membrane of endothelial cells (12) including those of the lung (13). Alteration inthe activity of this enzyme during development and as a result of exposure to cigarette smoke will be studied in Projects 2 and 3 of this program project.

It is now possible to isolate relatively pure populations of Type II cells (14) and the feasibility of studying these cells grown in culture has recently been demonstrated (15). Several groups have established cultures of functional active endothelial cells (e.g., 16,17,18). Although cultures of pulmonary endothelial cells have not yet been established, the existence of specific "markers" such as the enzyme that converts angiotensin I to angiotensin II (19) would allow identification of pulmonary endothelial cell in culture. Fibroblast cultures that synthesize and secrete procollagen are now routinely established from, for example, human skin biopsies (20) and the in vitro synthesis of collagen by minces of lung tissue has been studied (21).



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Therefore, we propose to examine the cellular basis of altered lung function associated with cigarette smoke and other environmental agents, utilizing isolated, functional alveolar cells maintained in cell culture.

Epidemiologic studies indicate that environmental and genetic factors influence the genesis of chronic obstructive lung disease. For example, there is an additive effect of smoking and exposure to "traffic pollution" in the development of disease (22). A clearcut example of the influence of genetic factors is the congenital deficiency in alpha$_1$-antitrypsin which results in a predisposition to emphysema that is exacerbated by cigarette smoking (23). The results of twin studies (24) imply that other more subtle, genetic factors may also have an appreciable effect. In the Report of the Surgeon General (25), the question is posed, "whether lesser degrees of genetically identifiable susceptibility interact with cigarette smoking to account for a significant proportion of the problem."

Experimental genetics in humans is not feasible and no animal models have yet been developed to investigate possible genetic factors in the genesis of chronic obstructive lung disease. However, the existence of many inbred (isogenic) strains of the mouse in which structural and/or functional variants of the lung may be found makes such an approach feasible. Morphometric variants are of prime interest, as shown by the work of Lynne Reid's group (26) which correlates the number of bronchial divisions with the development of early emphysema. Indeed, the hypothesis is posed in the general introduction to this program project that "certain patterns of lung geometry (may) predispose to accelerated pathogenesis of obstructive lung disease." To test this hypothesis we propose to seek congenital variants of lung geometry (number of different bronchial divisions) in different mouse strains and/or experimentally induce such variants, and study the effects of cigarette smoke on these mice.

Environmental influences during embryonic development, or during the extensive (27) post-natal maturation of the lung may, by altering the adult lung, also alter the susceptibility of this organ to environmental "insult" in later life. One such influence could be maternal smoking during pregnancy and/or post-natal maturation. Smoking has been implicated as a cause of increased infant mortality and morbidity (28) but little information is available on the effect of cigarette smoke on the developing lung. Therefore, we also propose to study the effects of cigarette smoke and other environmental factors on the morphological and morphometric development of the mouse lung.



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Principal Aims:

1. To study the effects of cigarette smoke on the growth and specific functions of isolated cell types from the alveolar wall grown in culture.
2. To determine whether mice with congenital or experimentally induced variations in lung morphometry respond differently to chronic inhalation of cigarette smoke.
3. To determine whether cigarette smoke influences pre or post-natal lung development and thus leads to altered lung properties.

General Experimental Design:

1. The animal model will be the laboratory mouse. The BALB/C strain will be used as the "standard" strain in all three designs, but other inbred (isogenic) mouse strains will be selected from those commercially available from Jackson Laboratories for studies in design 2. The BALB/C strain is also being used in the separate project being submitted to the Council by Dr. H. Herscowitz. Thus the results of his studies on the effects of cigarette smoke on alveolar macrophage function can be related to the results of the studies we propose.

2. Exposure to cigarette smoke.
Mice will be exposed to the smoke from University of Kentucky "reference" cigarette (1A1) in a prototype Walton Mark II horizontal smoke exposure machine. Cell and organ cultures will be exposed in the chamber recommended by Davies and Kistler (29) to allow adequte control of smoke mixing, temperature, humidity, etc. The dosage schedule will be determined in preliminary experiments after consultation with experts at the Council for Tobacco Research, U.S.A. Our aim is to duplicate the exposure of the "average" human smoker, as far as this may be possible.

3. Schematic presentation of the 3 major designs envisaged in this project are shown on the following 3 pages. In Design 1, pure populations of specific cell types of the alveolar wall from healthy mouse lung will be grown in culture and separately exposed in vitro to cigarette smoke. In Design 2, experimentally-induced and congenital variants in lung morphometry will be sought and the effects of such variants on lung metabolism and pathology studied. In Design 3, the effect of cigarette smoke on pre-and post-natal lung development will be studied by exposing pregnant females and neonates to smoke in vivo and by exposing organ cultures of developing lung to smoke in vitro.

It should be emphasized that the flow sheets outlining Designs I-III represent a concept of the experimental work we foresee. We do not propose that all of this work is to be accomplished within a five-year span. Rather, as certain



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avenues of approach seem especially promising, they will be emphasized. Other less fruitful options may be dropped completely. We would envisage that the flow sheets will change each year as work progresses.

We should further emphasize that Project III is closely linked to Projects I and II in that the experimental material generated in III is utilized in both I and II. Similar cross utilization of material occurs within Project III itself, as indicated by the dashed arrows.

4. Narrative description of individual experimental design.
a. Design I

Specific Background:

Investigation of the direct effects of cigarette smoke, or other environmental factors, on specific pulmonary cell types has been limited. However, a number of studies have been published on the direct effect of cigarette smoke, or its constituents, on the functions of the pulmonary macrophage (e.g. 30). A differential response of different lung cell types to cigarette smoke is implied by the pathological finding in different lung diseases that have been associated with cigarette smoking. For example, lung cancer is associated with stimulation of bronchial epithelial cells leading to hyperplasia and metaplasia. On the other hand, emphysema is characterized by degeneration of the aveolar walls. In vitro experiments using lung organ cultures (e.g. 32) have shown variations in cell response to cigarette smoke constituents, such as stimulation of epithelial proliferation and suppression of connective tissue cell type. In addition, the same cell type (ciliated bronchial epithelial cell) may exhibit altered susceptibility at different stages of development (32).

Previous in vitro studies have been directed towards detecting carcinogenic effects of components of cigarette smoke. Due to the high dosages used these studies do not provide satisfactory models for the chronic exposure of pulmonary cells to the levels of cigarette smoke, or air pollutants, found in the lungs of human smoker or non-smoker groups.

Procedures

a. Isolation of pulmonary cell types

Type II aveolar epithelial cells will be isolated by a modification of the procedure of Kikkawa and Yoneda (14) and used for direct biochemical analysis. The clonal isolation technique as used by Douglas and Kaighn (15) will be employed to derive cultures of functionally active type II cells. Cells from the peripheral tissue of healthy neonatal mouse lung will be disassociated by mild enzyme treatment and



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cultured at very low density to favor the growth of single cell clones. Clones of epithelioid morphology which also exhibit large cytoplasmic granules when examined by phase contrast microscopy will be analyzed for ultrastructural evidence of lamellar bodies (2) and biochemical evidence of dipalmitoyl lecithin synthesis (see project 2). Clones exhibiting both these type II cell "markers" will be used for in vitro experiments.

Pulmonary endothelial cell cultures will be obtained by selecting clones with "endothelioid" morphology (18) and specifically identified by the detection of the following recognized "markers"; 1) Weibel-Palade bodies as demonstrable by EM (33) and 2) ability of the cells to convert Angiotensin I to Angiotensin II demonstrable by the release of tritiated His-Leu from $^{3}$H-angiotensin I added to the culture (19,34).

Interstitial cell cultures will be derived by selecting "fibroblastic" clones and identified by demonstrating the presence of significant amounts of $^{3}$H-hydroxyproline in protein secreted into the medium after exposure of the culture to $^{3}$H-proline (20,35).

b. Exposure to cigarette smoke.

Replicate cultures of the individual cell types will be placed in the Davies and Kistler (29) chamber of the smoking machine and exposed to either 1) air, 2) whole cigarette smoke or 3) filtered cigarette smoke. Exposure will be arranged to be as closely analogous as possible to the exposure of cells in the distal airways of mice subjected to chronic smoking (see project I and Design II).

Dose response curves will be derived with respect to cell viability and plating (cloning) efficiency. Cultures exposed to non-toxic dosages will be examined for morphological and metabolic alterations (project 2) and, especially, any alterations in the expression of their "marker" functions.

Chronically exposed cultures will be analyzed periodically for possible progression of effects. Samples of such cultures will also be preserved in liquid nitrogen and thus will be available for further investigations.

DESIGN II

Specific Background

Breeding programs have resulted in the current availability of a large number of inbred (isogenic) mouse strains. Many anatomical variants in the various strains have been identified (36). No surveys for variants of lung morphometry have, so far, been undertaken.

With such excellent raw material and a practicable technique for morphometric screening, it is highly likely that strains showing congenital differences in the number of bronchial divisions can be identified for use in experimental study.

Chemicals have been used to modify the degree of bronchial branching in organ cultures of embryonic lungs (37,38). As there is extensive branching during postnatal lung maturation (27) the application of such chemicals to the lungs of neonatal mice may allow modification of lung maturation so as to provide morphometric variants with identical genetic



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backgrounds.

Procedures

1. Mice of various inbred strains will be the subjects for lung morphometry described in the general methods section. Preliminary studies will establish the age ( 21 days; see ref. 27) at which all strains to be analyzed have completed post-natal lung maturation. Lungs of mice at this age (at least 3 litters, with at least 3 mice of each sex) will be analyzed to determine the average number of bronchial divisions and the variation a) between sexes, b) among mice of the same sex and c) between litters. Because of the isogenic nature of each strain, the latter two sources of variation should be minimal. The sample size may be altered depending upon the results of the preliminary statistical evaluation.

2. Newborn animals will be exposed to papaverine (37), cytochalasin (see discussion by Wessells et al., 39) or proteolytic enzymes (40) which may be expected to impede or prevent subsequent bifurcation of developing lung epithelium. The method of exposure will be by allowing animals to inhale an aerosol containing the agent. The use of an inbred strain plus the prior establishment of intra-strain variance, makes use of litter mates of treated animals as controls quite valid.

3. From procedures 1) and/or 2) we expect to have groups of mice with lungs having low, intermediate and high numbers of bronchial divisions. Each group will be divided into: a) mice to be chronically exposed to cigarette smoke for a period of at least 6 months; and b) controls similarly treated but exposed to air only. At the end of this period analysis of lung function (project 1) and lung metablism (project 2) will be performed. The possible differential effects of cigarette smoke on mice with different lung morphometry will be ascertained.

DESIGN III

Specific Background

There is at present no integrated compilation of developmetnal data on the lung for any one animal model in terms of morphological, mophometric, physiological and biochemical parameters. The purpose of this part of Project 3 is to obtain such a profile of the properties of developing lung in BALBIC mice, and to determine whether exposure to cigarette smoke alters this development. As considerable and significant changes occur during the post-natal maturation of the lung (27), our study will encompass both pre- and postnatal development.

Further, fetal lungs from the three groups to be studied (see Procedures) will be explanted in organ culture at various developmental stages. Organ culture techniques are well established (42). Fetal lung has been grown in organ culture by Wolff,[41] Taderera,[44] and Chan et al[45] among others. The organ culture system provides for the use of conditions which are well defined by comparison to those extant in vivo. Thus, any influence of vitamins or hormones on response of lung tissue to smoke exposure could be



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more directly measured. Further, in organ culture, three-dimensional relationships of tissues are maintained to a considerable degree. If bronchial branching is impeded or prevented, is there a modification of response to smoke exposure? Is the pattern of lung branching in vitro changed in lungs from fetuses whose mothers have been chronically or acutely exposed to smoke? Does smoke itself directly affect the branching activity of lung buds already in vitro? The in vitro system permits us to perform experimental manipulations which would be difficult indeed if attempted in vivo.

Another means of isolating fetal lung tissue from its normal environment to make it accessible to experimental manipulation is to graft it to the chick embryo chorioallantoic membrane (CAM). Grafts of embryonic organs to the CAM of the chick embryo become vascularized and continue to develop with good maintanance of organ relationships. This technique has been used for years and is a simple one (reviewed in ref. 46). Mammalian organs are generally well maintained on the CAM. Larger rudiments can be grown than will survive in organ culture. Here then is a system in which the vascularized lung can be directly exposed to smoke. Morphometric analysis is quite feasible here. CAM grafts can also be used to monitor hormonal influences. Use of pituitaryless embryos (47) as hosts for the grafts provides a hormone deficient environment for the lung. Replacement therapy can be used to isolated individual endocrine effects.

Fetal lung grown either as a graft or in culture can be directly exposed to a branching inhibitor (in order to determine whether subsequent exposure to smoke results in an accentuated change in lung properties). However, the precise nature of the experments to be performed in organ culture or with CAM grafts will depend upon the in vivo findings in all three projects during the first 2 years of the program.

It should be reiterated here that Design III represents a large concept of experimental approach to the problem of analyzing lung development and some of the factors affecting this development. We do not imply that this entire Design will be accomplished within five years. Indeed certain lines of approach may well be found impractical and others not outlined here more meaningful. Thus, while the aims of Design III remain, the methods or lines of approach may vary.

Procedures

1. To study the possible effects of exposure to cigarette smoke on prenatal lung development, female BALB/C mice will be divided into three groups: chronic, acute and control. Chronic animals will be exposed to cigarette smoke for 21 days prior to mating. Exposure will continue throughout pregnancy. Acute animals will be exposed to smoke from day 1 of pregnancy and every day throughout pregnancy. Control animals will be handled in the same manner as experimentals but will be exposed only to air in the smoking apparatus.

It is recognized that every environmental parameter cannot be considered in every experimental design. However,



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a few pilot studies will serve to decide whether total food intake should be monitored. Hence, one group of both control and acute animals will be given a diet corresponding to the amount consumed by the chronic group of animals. This dietary regime will commence in control and acute animals at the same time as the chronic group commences exposure to smoke. The results will help determine whether diet should be regulated in control and acute groups in later experments. This may be necessary if it is found that decreased food intake in the chronic group is responsible for any measured differences in the other groups.

Pregnancies will be terminated in three animals of each group on days 5, 12, and 17 (assuming a 19-day gestation). Data will be collected in the following format:

| | Chronic | | | Acute | | | Control | | |
|---|---|---|---|---|---|---|---|---|---|
| Day | 5 | 12 | 17 | 5 | 12 | 17 | 5 | 12 | 17 |
| # resoptions/horn | | | | | | | | | |
| # implants/horn | | | | | | | | | |
| position of implants in horn | | | | | | | | | |
| gross abnormalities | | | | | | | | | |
| wet wt. of each fetus* | | | | | | | | | |
| DNA/fetus* | | | | | | | | | |

* These measurements will be recorded in terms of site of implantation in horn. DNA will be measured according to Schmidt (Colowick & Kaplan, Methods in Enzymology, Vol 3).

The data accumulated in this part of the design will establish a reliable baseline of fetal properties. This is important in understanding the variability in response to be expected from one experiment to the next. The following questions about fetal response to maternal smoke inhalation should be answered: a) do chronically exposed mothers produce litters with characteristics different from those of acutely exposed or control mothers (see methodology section)? b) Can severity of response be related to cumulative exposure time?

Lungs from fetuses of different and from newborn mice will be examined in terms of their morphological, morphometric (especially bronchial divisions) and metabolic (see Project 2) properties.

2. The effects of postnatal exposure to cigarette smoke on lung maturation will be studied by exposing litters of newborn mice to smoke daily during the period of maturation (23 days, ref. 27). At the end of this period the lungs of control and smoke-exposed mice will be analysed in terms of morphological, morphometric and metabolic properties.

3. To experimentally analyse factors involved in possible changes in fetal lung development associated with in vivo exposure to cigarette smoke, portions of fetal lung at various stages of development will be established in organ culture and/or as grafts to chick chorioallantoic membrane (CAM).

a) Organ culture techniques have been routinely used in our laboatory and technical details can be found in the references cited above. In brief, embryonic lung rudiments will be removed from the fetus under aseptic conditions, and placed in a culture dish, either on a semi-solid sub-



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strate such as agar mixed with nutrient medium, or on a 'raft' floating on a liquid nutrient medium. The medium selected will be one that discourages outgrowth of cells from the organ explant and hence tends to maintain organ integrity. Development of the organ explants will be followed using the morphological and biochemical criteria described in Projects 1 and 2. Such cultures can be directly exposed to smoke and changes in lung properties attributable to smoke monitored.

b) Similarly, we have used the CAM grafting technique routinely. A small window will be cut through the shell and shell membrane of a 7-8 day old embryonated hen's egg. Lung buds removed from fetuses asceptically will be placed near a larger blood vessel on the CAM. The window will be sealed (scotch tape serves well) and the egg incubated until the graft has become well vascularized. For smoke exposure, or addition of chemicals, the window will be temporarily re-opened. If long term experiments are desired (longer than 10-11 days, at which time hatching of the chick would be imminent!), the graft will be removed at day 10-11 and re-passaged on a new 7-8 day old host.

1st Year: During the first year our major efforts will be devoted to 1) establishing cultures of functionally active alveolar cell types (Design I) and 2) establishing "baseline" data on the fetal developemnt of BALB/C mouse lungs and the influence of smoking on this development (Design III). We also plan to standardize the procedures for morphometric analysis of bronchial divisions on BALB/C mice (Design II).

2nd Year: We plan to undertake the experimental portion of Design I, analysing the effects of cigarette smoke on cultures of different cell types. When the analysis of fetal lung development is completed we will go on to study post-natal lung maturation and the effects of exposure to cigarette smoke upon this maturation (Design III, cont.). During the second year we also expect to be able to begin the morphometric survey of lungs in different inbred mouse strains (Design II).

3rd Year: We expect to concentrate on finishing the morphometric survey. If the first 4 inbred lines surveyed do not show any significant differnces in number of bronchial divisions we will turn to chemical modification of branching (Design II, part b) to obtain variants. The data on normal lung development that has been accumulated (from Projects 1,2 and 3) by this time should provide a good base for judging the effects of these chemicals on lung development.

4th Year: We hope to have groups of mice with lungs having respectively high, intermediate and low numbers of bronchial divisions. Chronic smoking of these mice will begin. During the fourth year we would also expect to use the cell cultures and/or organ cultures as models for experimentally testing the effects of other agents (e.g. vitamins, hormones, environmental pollutants) on lung cell function and/or lung developemnt in absence of, or presence of, cigarette smoke. The nature of these experiments will depend upon hypotheses generated from the data accumulated in all three projects of this program, as well as that reported by other investigators.

5th Year: Analysis of whether differences in the



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number of bronchial divisions alters the susceptability of mice to chronic cigarette smoking should be possible. The results from Design II should provide more hypotheses to be tested in the "model" system that is most appropriate.

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ELECTRON MICROSCOPIC PROCEDURES

The methodology described below will be used in all three projects:

A. Ultrastructural Methods. Tissue samples from lungs in vivo, perfused lungs and in vitro cultured cells will be fixed in an aldehyde mixture (1) for two hours at 4°C and washed overnight in an appropriate buffer. Following the post-fixation in one percent osmium tetroxide, the samples will be dehydrated, embedded in Epon and sectioned with a diamond knife in 300-600 A° thin sections. Some grids will be treated conventionally with heavy metals, whereas the other grids will be processed according to phosphotungstic acid (10% aqueous solution, pH 1.5) procedure of Kay (2) and silver tetraphenylpophinesolfonate technique of Albert and Fleisbher (3) for demonstration for collagen and elastin fibers respectively.

B. Autoradiographic Methods (4). Tissue samples from lungs in vivo, perfused lungs and in vitro cultured cells will be fixed in a 2% osmium tetroxide buffer with veronal actate to pH 7.2 for two hours at 4°C and washed overnight in an appropnate buffer. Following the block staining in 1% uranyl actate, dehydration and embedding in Epon, tissue samples will be sectioned with a diamond knife in 600-900 A°thin sections and mounted on grids previously coated with collodion film baked by a thin carbon layer. The grids will be exposed to the photosensitive emulsion (Ilford L-4) diluted one to four with distilled water for approximately six months, developed in Microdal X for 5 minutes, washed, fixed in Kodak rapid fixer for 5 minutes and washed again in running and distilled water for 5 and 2 minutes respectively.

Electron microscopy and photogrphy will be carried out with the aid of an AEI-EM 8 electron microscope.

C. Preparation of Tissue for Scaning Electron Microscopy: Fixation in formaldehyde-glutaraldehyde 3% buffered to pH 7.2 with sodium cacodylate 0.1 M for 2 hours at 4°C will be followed by washing in buffer for up to 24 hours. The tissue will be then fixed in aqueous solution of 2% OsO4 for 1-2 hours and dehydrated by the critical point dry technique. Coating with a layer of ca 200A° of gold-parllodium will be the final step before observation with Etec Autoscane-B Scaning Electron Microscope.

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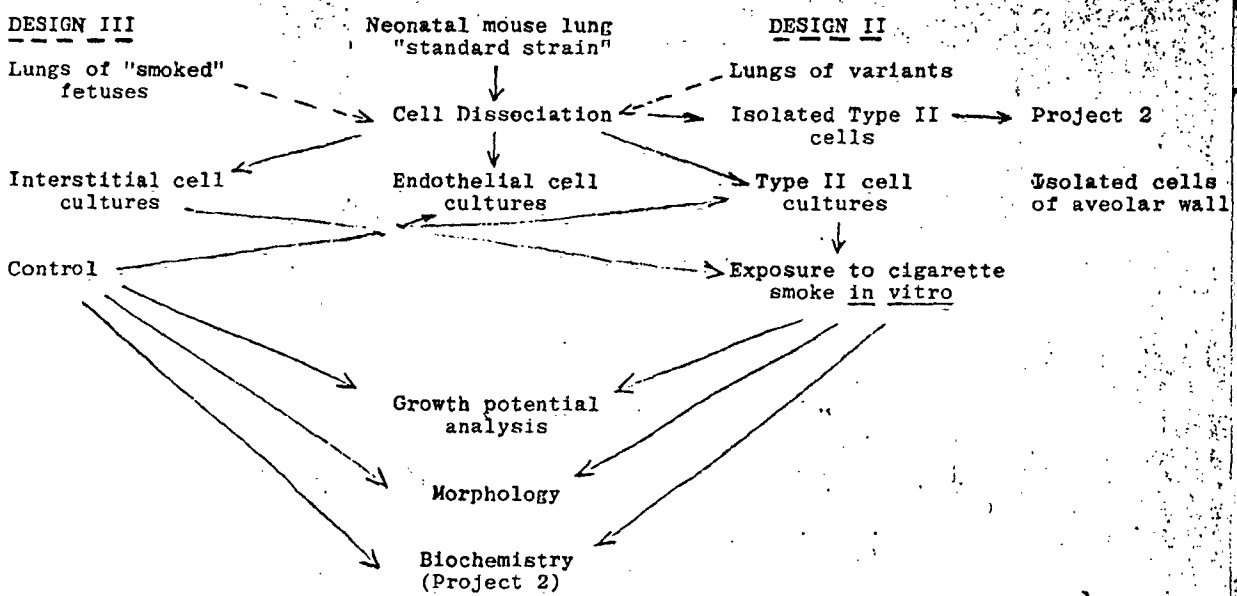
P. HAMOSH M.D.

PROJECT 3

DESIGN I




P. HAMOSH M.D.

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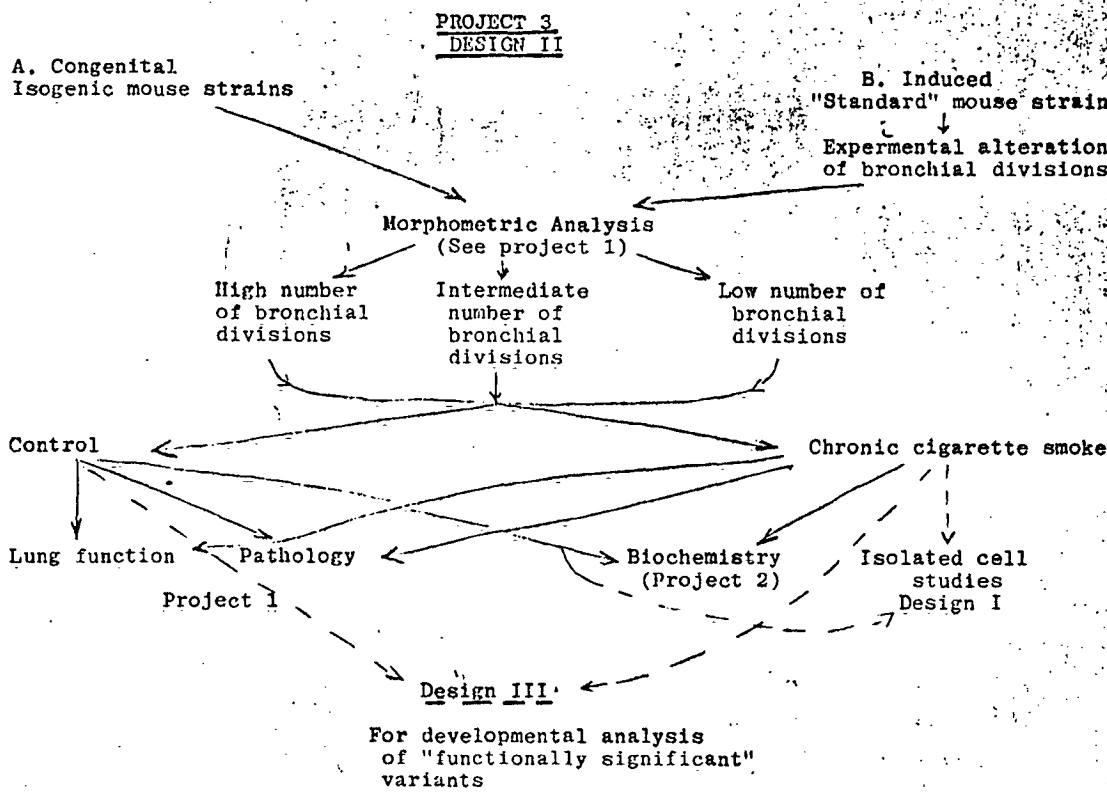


P. HAMOSH M.D.

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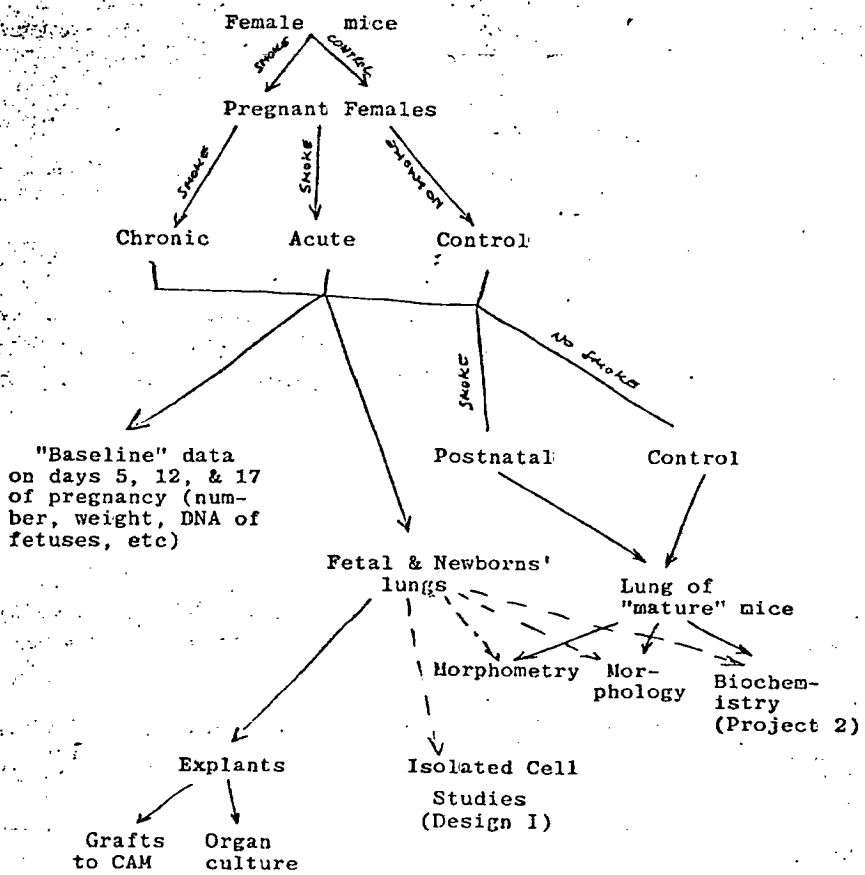
## DESIGN III



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First Year Budget
Project 3.

P. HAMOSH M.D.

3.

13. Budget: (1st year)

| | % time | Amount |
|---|---|---|
| **A. Salaries (Personnel by names)** | | |
| Professional | | |
| Jean Rew Wrathall Ph.D. | 50% | REDACTED |
| Technical | | |
| To Be Hired | 100% | R |
| All Salaries Include 18.35% Fringe Benefits (16.7% 1/1/76-6/30/76 and 20% 7/1/76-12/31/76) | Sub-Total | ~~REDACTED~~ |
| **B. Consumable Supplies (list by categories)** | | |
| Chemicals | | 2,500 |
| Glassware and Disposables | | 2,500 |
| Isotopes | | 2,500 |
| Animals and Care | | 1,500 |
| Surgical Instruments | | 1,000 |
| | Sub-Total | 10,000 |
| **C. Other Expenses (itemize)** | | |
| Computer Time | | 4,500 |
| Travel | | 1,500 |
| | Sub-Total | 6,000 |
| **D. Permanent Equipment (itemize)** | | |
| Laminar Flow Hood | | 1,800 |
| Co2 Incubator | | 1,300 |
| Elutriation Rotor | | 6,500 |
| Gradient Mixer and Pump | | 4,700 |
| | | 14,300 |
| **E. Overhead (15% of A+B+C)** | Total | 52,722 |

Overhead to be negotiated at 15% or higher of A,B, & C

The distribution of effort by all investigators is presented in Figure 2

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FACILITIES AVAILABLE: (Listed by Investigators)

Paul Hamosh, M. D. Department of Physiology and Biophysics

Space: Office, Secretary's office, laboratory for human studies, all purpose laboratory and laboratory for animal experiments (total area approximately 1000 square feet).

Space Shared: Departmental workshop, dark room, library and common office equipment room (copy machine, computer terminals and calculators).

Equipment:

Two 8 channel recorders - Hewlett-Packard (HP)

One x/y recorder HP

One 4 channel FM tape recorder HP

Four oscilloscopes, Tektronix

Twenty different amplifiers, HP, Validyne, Lexington, Tektronix

Two body plethysmographs with electronics

One total respiratory resistance unit, Lexington

One respiratory mechanics computer, Buxco

One blood gas analyzer, Corning

One bicycle ergometer, Quinton

Twenty transducers

Assorted animal respirators and plethysmographs complete electronic testing equipment, infusion and peristaltic pumps, etc.

Use of the facilities of the National Biomedical Research Foundation on a pay for services basis: an IBM 360/44 computer with all peripherals (see appended description).

Margit Hamosh, Ph.D.

Space: Office and Biochemistry Laboratory (approximately 250 square feet)

Space Shared: Additional laboratory space, cold room and conference room.

Equipment:

One spectrophotometer (Beckman 24)

One tissumizer (Tekmar)

One ultrasonic sonifier (Laboratory Supply Co.)

One driblock (Tecam)

IEC International centrifuge, Duenoff shaker, ovens, pumps, mixers, stirrers, chromatography equipment, etc.



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Dr. Jean Wrathall, Ph.D.

Space: Tissue culture suite -approximately 800 square feet of space including 2 small "clean" rooms for aseptic transfers, a walk-in cold box, a small room with a chemical hood and 2 large rooms.

Equipment:

One dishwaser
One sterilizing oven
One Hotpak $CO_2$ forced-air incubator
One convection heated incubator
One table top clinicalcentrifuge
One Leitz microscope with objectives including oil immersion
One Olympus inverted microscope with conventional and phase contrast optics
One Cyrenco liquid nitrogen freezer
Two dissecting microscopes
One water bath
One magnetic stirrer
An autoclave is available in the Microbiology Department on the floor above.
Di-ionized water of satisfactory quality is available from the Microbiology Dept.

Dr. Gerald Goeringer, Ph.D.

Office Space:144 square feet

Laboratory space: 350 square feet

Equipment:

One Spinco Model L-4 ultracentrifuge with SW-65, SW-25 and 60 rotors
One International Model B-20 refrigerated centrifuge with 870 and swing-out rotors
One International Model H-12 table centrifuge with fixed and wing-out heads
One Zeiss model PMQII spectrophotometer
One ISCO UV Analyzer (in need of repair), Model UA-2
One EC 10 ml 20-tube counter-current train
One EC 20 ml 20-tube counter-current train
One EC Disc electrophoresis apparatus
One Buchler Model 3-1014A regulated voltage, current power supply
One Porter Blum Model MT-1 Ultramicrotome
One LKB Ultrorac Fraction Collector (in need of modification)
One Buchi Flash evaporator
One Precision Scientific Co. full view tissue culture incubator
One Labconco tissue culture hood



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Goeringer continued

One New Brunswick controlled environment shaking incubator with 1 platform

One Zeiss photomicroscope II, Nomarski optics

One Zeiss Phase contrast microscope

One Heat systems Model W140 Ultrasonicator with 1 probe

One Sigmamotor peristaltic pump

One Photovolt Digicord pH meter

One Mettler H-16 Balance

One Mettler microbalance

One Mettler P-1000 toploading balance

One AO Stereomicroscope, Double head

One vacuum oven, vacuum pump, Virtis Lyophilizer (modified) (in need of repair)

One drying oven

Branislav Vidic, S.D.

Light Microscopy: Fully-equipped laboratory for processing of tissues (paraffin oven, hot plate), sectioning (microtome) and light microscopy (Zeiss binocular microscope with incorporated camera) are available in the Department of Anatomy.

Electron Microscopy: Fully-equipped laboratory for preparation of tissues, sectioning (diamond knives, LKB-ultramicrotome) and electron microscopy, transmission (AEI-EM 801) and scanning (ETEC AUTOSCAN B-1) are available on a part-time basis in the Department of Anatomy.

Major Facilities Required:

One Scintillation Counter

Two Smoking Machines

(The latter will be provided by the CTR on a lease basis).



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## Justification of Budgets

### Project #1

1. Dr. Da Silva is joining on a fulltime basis the project in July 1976. Until then his support is assured from other sources. Projects A and D.
2. Ms. Linda Cooper has worked for the past year with project CTR #878. Project A and D.
3. A technician for histology to handle projects B and C, and projects 2 and 3 is included in full.
4. A second technician will be responsible to provide field support in project A and instrumentation support in all other projects.
5. Computer time is rated for 100 hours at $90. - (IBM 360/44).
6. Equipment: The system for the field station is an estimated price. We have several systems under investigation. A firm quote will be available later this year.
7. Other equipment is self explanatory and the estimates are based on previous work done for use at Johns Hopkins and Harvard Universities, whose craftsmen we frequently utilize.

### Project #2

1. Dr. Margit Hamosh draws tentatively her entire support from this project. If additional sources now pending become available, this will be reduced.
2. Post doctoral fellows are being interviewed for this position, requiring some skill and experience.
3. A technician (probably Ms. Ruth Avigan) will provide technical support for this work.
4. Repair and Maintainance for service contracts on major equipment.
5. A new centrifuge is needed to replace an ailing international.

### Project #3

1. Dr. Jean Wrathall will draw 50% of her support for the entire year.
2. The technician to be hired will assist in tissue culture work and in experiments performed by Drs. Wrathall and Goeringer.
3. Computer time is for morphometric work (50 hours).
4. Equipment. The hood and incubator are essential to upgrade the tissue culture suite. The rotor and mixer are used in cell separation.

### Central Service and Facility

1. Part of Dr. Hamosh's salary is requested due to institutional guidelines.
2. The administrative assistant is co-ordinating all clerical work and bookkeeping and representing the program project vis à vis the administrative branches of the University (sponsored programs, purchasing, payroll, library, etc.).
3. The technician has dual function a) co-ordination of animal facilities, specimen flow and equipment maintainance and representation of the project vis à vis the technical branches of the University (housekeepping, engineering, supplies, etc.). b) provide "reserve" manpower, where most needed.

Equipment:

A scintillation counter is essential for the whole goup



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(and Dr. Herscowitz). Presently the group is utilizing counter available by courtesy of their owners in Depts. of Physiology Pharmacology, Microbiology and Nuclear Medicine, with increasing volume of work, these arrangements will be insufficient.

Budgetary Considerations For Future Years

Whereas equipment expenditures are expected to decline in future years of support, expenses for salaries and supplies are expected to increase due to inflation and possible need of expansion. Budgets will be submitted for each fiscal year 6 months in advance. It is safe to assume that budget for the second year will remain about the same level as the first year budget and then increase at a rate of 5 to 10 percent.



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SECTION II – PRIVILEGED COMMUNICATION

## BIOGRAPHICAL SKETCH

(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

| NAME | TITLE | BIRTHDATE (Mo., Day, Yr.) |
|---|---|---|
| Hamosh, Paul, M.D. | Assoc. Professor of Physiology, Biophysics & Medicine | REDACTED |
| PLACE OF BIRTH (City, State, Country) | PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa) REDACTED | SEX ☒ Male ☐ Female |

REDACTED EDUCATION (Begin with baccalaureate training and include postdoctoral)

| INSTITUTION AND LOCATION | DEGREE | YEAR CONFERRED | SCIENTIFIC FIELD |
|---|---|---|---|
| Hadassa, Hebrew University Medical School Jerusalem, Israel | M.D. | R | |

HONORS

| MAJOR RESEARCH INTEREST | ROLE IN PROPOSED PROJECT |
|---|---|
| Lung Mechanics and Metabolism | Responsible Investigator |

RESEARCH SUPPORT (See instructions)

Principal Inv.: "The Effect of Smoking on the Small Airways". Grant #87 Council for Tobacco Research, Inc., U.S.A. $28,670 p/a (1/1/73 - 12/31/ effort-20%

Co-principal Inv. (with Dr. B. Vidic): "The Effect of Tobacco Smoke on Lung Metabolism." Grant # 936 Council for Tobacco Research, Inc., U.S.A. $40,000 p/a (1/1/74 - 12/31/76) effort-20%

Principal Inv.: "Chylomicrons and Atherosclerosis." Grant #802 Washingt Heart Ass. $11,615 (7/1/75 - 6/30/76) effort -10%

RESEARCH AND/OR PROFESSIONAL EXPERIENCE (Starting with present position, list training and experience relevant to area of project. Li or most representative publications. Do not exceed 3 pages for each individual.)

1975– Associate Professor of Physiology and Biophysics and Medicine Georgetown University Medical School, Washington, D.C.

1973-date Senior Cancer Research Internist, Veterans Administration Hospital/National Cancer Institute, Washington, D.C. (Part-ti

1973-date Special Lecturer in Child Health Care and Development and Behaviorial Sciences, George Washington University Medical Schoo Washington, D.C.

1973-1975 Assistant Professor of Medicine, Georgetown University Medica School, Washington, D.C.

1972-1975 Assistant Professor of Physiology and Biophysics, Georgetown University Medical School, Washington, D.C.

1970-1972 Director, Pulmonary Physiology Laboratory, Veterans Administration Hospital, Washington, D.C.

1970-1972 Assistant Professor of Medicine, George Washington University Medical School, Washington, D.C.

1968-1970 Research Associate, Veterans Administration Hospital, Washington, D.C.



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Paul Hamosh Curriculum Vitae (Continued)

1968–1970 Lecturer in Physiology, Georgetown University Medical Schoo Washington, D.C

1966–1968 NIH Trainee in Cardio-Pulmonary Physiology, Department of Medicine, Georgetown University Medical School and Veterans Administration Hospital, Washington, D.C.

1963–1965 Department of Medicine B, "Ichilow" Municipal Hospital, Tel Aviv, Israel

1957–1963 Internship and Residency in Pathology, Medicine and Chest Diseases, Hadassa University Hospital, Jerusalem, Israel

PARTIAL LIST OF PUBLICATIONS

Dreyfuss, F., Hamosh, P. A coronary disease study among Cochin Jews in Israel. Am. J. Med. Sciences 240:769, 1960.

Dreyfuss, F., Hamosh, P., Adam, Y.G., Kallner, B. Coronary heart disease and hypertension among Jews immigrated to Israel from the Atla Mountain Region of North Africa. Am. Heart J. 62:470, 1961.

Hamosh, M., Hamosh, P., Bar-Maor, J.A., Cohen, N. Fatty-acid metaboli by human adipose tissues. J. Clin. Invest. 42:1648, 1963.

Zlotnich, A., Ramot, B., Hamosh, P. Hemoglobin-H disease with persistent hemoglobin "Bart's" in a Jewish family of Aleppo-Urfalian ancestry. Israel Med. J. 23:57, 1964.

Hamosh, P., Ramot, B., Molho, M. Primary hypoventilation syndrome of central origin. Israel J. Med. Science 2:448, 1966.

Ramot, B., Hamosh, P., Loeventhal, M.et al: Disseminated eosinophilic collagen disease. New Istanbul Contrib. Clin. Sc. 9:22, 1967.

Hamosh, P., Luchsinger, P.C. Maximum expiratory flow in isolated liquid-filled lungs. J. Appl. Physiol. 25:495, 1968.

Cohn, J.N., Khatri, I.N., Hamosh, P. Bedside catheterization of the left ventricle in shock: a valuable diagnostic and therapeutic guide. Am. J. Cardiol. 25:66, 1970.

Hamosh, P., Luchsinger, P.C. Respiratory mechanics and gas exchange i the squatting position. Am. Rev. Resp. Dis. 102:112, 1970.

Hamosh, P., Cohn, J.N. Left ventricular function in acute myocardial infarction. J. Clin. Invest. 50:523, 1971.

Hamosh, P., Broder, M.I., Engelman, K., Cohn, J.N., Freis, E.D. Systo lic time intervals in acute myocardial infarction. Circulation 45:37 1972.

Gacad, G., Hamosh, P. The lung in ankylosing spondylitis. Am. Rev. Resp. Dis. 107:286, 1973.



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Partial List of Publications, Continued

Da Silva, A.M.T., Hamosh, P. The effect of smoking a single cigarette on the small airways. J. Appl. Physiol. 34:361, 1973.

Hamosh, P., Da Silva, A.M.T. Supine hypoxemia and erythocytosis due to airway closure at low lung volumes. Am. J. Med. 55:80, 1973.

Hamosh, P., Da Silva, A.M.T. Air bolus method compared to single breath method for determination of closing volume. Am. Rev. Resp. Dis. 110: 518, 1974.

Hamosh, M., Hamosh, P. Lipoprotein lipase in rat lung, the effect of fasting. Biochim. Biophys. Acta. 380:132, 1975.

Hamosh, M., Hamosh, P. Effect of estrogen on lipoprotein lipase activity of rat adipose tissue. J. Clin. Invest. 55:1132, 1975.

Vidic, B., Hamosh, M., and Hamosh, P. Mucopolysacchrides in the pulmonary surfactant hypophase. An electron microscopical study of rat lungs treated with ruthenium red. J. Cell Biol. Submitted for publication.

Hamosh, P. The effect of shearing stress on the bronchial mucosa. Am. Rev. Resp. Dis. 109:694, 1974. (Abstract)

Vidic, B., Hamosh, M., Blunda, M., Hamosh, P. Effect of ventilation on uptake of $^3$H-palmitate by isolated perfused rat lung. J. Cell Biol. 63:359a, 1974. (Abstract)



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SECTION II – PRIVILEGED COMMUNICATION

## BIOGRAPHICAL SKETCH

*(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)*

| NAME | TITLE | BIRTHDATE (Mo., Day, Yr.) |
|---|---|---|
| Hamosh, Margit, M. Sc., Ph.D. | Assistant Professor | REDACTED |
| **PLACE OF BIRTH** *(City, State, Country)* | **PRESENT NATIONALITY** *(If non-U.S. citizen, indicate kind of visa and expiration date)* | **SEX** |
| REDACTED | REDACTED | ☐ Male ☒ Female |

**EDUCATION** *(Begin with baccalaureate training and include postdoctoral)*

| INSTITUTION AND LOCATION | DEGREE | YEAR CONFERRED | SCIENTIFIC FIELD |
|---|---|---|---|
| Hadassa, Hebrew University Medical School Jerusalem, Israel | M. Sc. Ph. D. | R | Microbiology Biochemistry |

**HONORS**

M. Sc. Summa Cum Laude

| MAJOR RESEARCH INTEREST | ROLE IN PROPOSED PROJECT |
|---|---|
| Lipid Metabolism | Principal Investigator |

**RESEARCH SUPPORT** *(See instructions)*

Co-Inv. : "The Effect of Tobacco Smoke on Lung Metabolism." Grant # 936 Council for Tobacco Research, Inc., U.S.A. $40,000 p/a (1/1/74 - 12/31/76)

Princ. Inv.: "The Effect of Estrogens on Human Adipose Tissue Lipoprotei Lipase Activity." Washington Heart Association # 690. $10,350 (1/1/75 - 12/31/75) effort - 10%

**RESEARCH AND/OR PROFESSIONAL EXPERIENCE** *(Starting with present position, list training and experience relevant to area of project. Li or most representative publications. Do not exceed 3 pages for each individual.)*

| | |
|---|---|
| 1975– | Assistant Professor, Dept. of Anatomy, Georgetown University Medical School |
| 1974-1975 | Research Associate, Dept. of Anatomy, Georgetown University Medical School |
| 1967-1974 | Visiting Scientist, NIAMDD, Section on Endocrinology (R.C. Sc |
| 1965-1967 | Sabbatical leave, Visiting Scientist, NIMH, General & Comparative Biochem. (S. Kaufman) |
| 1964-1965 | Lecturer in Biochemistry, Hadassa, H.U. Medical School, Dept. of Biochemistry |
| 1961-1964 | Instructor in Biochemistry, Hadassa, H.U. Medical School |
| 1959-1961 | Post-Doctoral Fellow in Biochemistry, Hadassa, H.U. Medical School |
| 1956-1959 | Graduate Fellow in Biochemistry, Hadassa, H.U. Medical School |

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PARTIAL LIST OF PUBLICATIONS

M. Hamosh: Recent aspects on the pathogenesis of fever. Harefuah 58:376, 1960.

M. Hamosh and B. Shapiro: The localization of bacterial endotoxin in the organism. Dapim Refuiim 19:3, 1960.

M. Hamosh and B. Shapiro: The mechanism of glycogenolytic action of endotoxin. Brit. J. Exp. Path. 41:372, 1960.

E. Wertheimer, M. Hamosh and E. Shafrir: Factors affecting fat mobilization from adipose tissue. Am. J. Clin. Nutr. 8:705, 1960.

M. Hamosh and B. Shapiro: Lipid release by liver slices. Am. J. Physiol. 201:1030, 1961.

M. Hamosh, P. Hamosh, A. Bar-Maor and H. Cohen: Fatty acid metabolism by human adipose tissue. J. Clin. Invest. 42:1648, 1963.

M. Hamosh, R. Atia and B. Shapiro: Fatty acid uptake and esterification by fish muscle. J. Food Sci. 31:146-150, 1966.

M. Hamosh, M. Lesch, J. Baron and S. Kaufman: Enhanced protein synthesis in a cell-free system from hypertrophied skeletal muscle. Science 157:935, 1967.

M. Lesch, W. W. Parmely, M. Hamosh, S. Kaufman, E. H. Sonnenblick: The effects of acute hypertrophy on the contractile properties of skeletal muscle. Am. J. Physiol. 214:685, 1968.

Margit Hamosh and Robert O. Scow: Plasma triglyceride and mammary and adipose tissue lipoprotein lipase in pregnant and lactating rats. Yugoslav. Physiol. Pharmacol. Acta 6:169, 1970.

M. Hamosh, T. R. Clary, S. S. Chernick and R. O. Scow: Lipoprotein lipase activity of adipose and mammary tissue and plasma triglyceride in pregnant and lactating rats. Biochim. Biophys. Acta 210:473, 1970

M. Hamosh and R. O. Scow: Lipoprotein lipase activity in guinea pig and rat milk. Biochim. Biophys. Acta 231:283, 1971.

R. O. Scow, M. Hamosh, E. J. Blanchette-Mackie and A. J. Evans: Uptak of blood triglyceride by various tissues. Lipids 7:497, 1972.

M. Hamosh and A. J. Evans: Lipoprotein lipase activity and triglyceride uptake in perfused adipose tissue. Fed. Proc. 31:297, 1972.

M. Hamosh and R. O. Scow: Lingual lipase and its role in the digestio of dietary lipid. J. Clin. Invest. 52:88, 1973.

O. Zinder, M. Hamosh, T. R. Clary-Fleck and R. O. Scow: Effect of



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prolactin on lipoprotein lipase in mammary gland and adipose tissue of rats. Am. J. Physiol. 226:744, 1974.

M. Hamosh and P. Hamosh: Lipoprotein lipase in rat lung. Fed. Proc. 33:346, 1974.

W. A. Burns, M. J. Mathews, M. Hamosh, G. Vender Weide, and F. B. Johnson: Lipase secreting acinar cell carcinoma of the pancreas with polyarthropathy: A light and electron microscopic histochemical and biochemical study. Cancer. 33:1002, 1974.

B. Vidic, M. Hamosh, M. Blunda and P. Hamosh: Effect of ventilation on uptake of $^{3}H$-palmitate by isolated, perfused rat lung. J. Cell Biol. 63:359A, 1974.

M. Hamosh, R. O. Scow, H. L. Klaeveman and R. O. Wolf: Role of pharyngeal lipase in lipid digestion in man. Clin. Res. 22:360A, 1974.

M. Hamosh and P. Hamosh: Lipoprotein lipase in rat lung, the effect of fasting. Biochim. Biophys. Acta 380:132, 1975.

M. Hamosh, H. L. Klaeveman, R. O. Wolf, and R. O. Scow: Pharyngeal lipase and digestion of dietary triglyceride in man. J. Clin. Invest. 55:908, 1975.

M. Hamosh and P. Hamosh: Effect of estrogen on lipoprotein lipase activity of rat adipose tissue. J. Clin. Invest. 55:1132, 1975.

M. Hamosh and A. R. Hand: Development of secretory activity in rat lingual serous glands. J. Dent. Res. 54:168, 1975.

## CHAPTERS IN BOOKS

Margit Hamosh and Robert O. Scow: Plasma triglyceride and lipoprotein lipase activity in pregnant and lactating rats. Nutrition Proc. VIIIth International Congress of Nutrition, Prague 1969, Ed. J. Masek et al., Excerpta Medica, ICS No. 213, 207-209, 1970.

R. O. Scow, E. J. Blanchette-Mackie, M. Hamosh and A. J. Evans: Catabolism of plasma lipoproteins, in "Structure, metabolism, and clinical aspects of lipoproteins of blood." Wissenschaftliche Veroffentlichung der Deutschen Gesellschaft fur Ernahrung 23:100-114, 1973.

R. O. Scow, C. L. Mendelson, O. Zinder, M. Hamosh and E. J. Blanchette-Mackie: Role of lipoprotein lipase in the delivery of dietary fatty acids to lactating mammary tissue, in Dietary Lipids and Post Natal Development, Galli, C., Jacini, G. and Pecile, A. (Eds.) Raven Press, 91-114, 1973.

M. Hamosh and R. O. Scow: Lingual lipase, in Fourth Symposium on Oral Sensation and Perception, Ed. J. F. Bosma, 311-322, Fogarty International Center Proceedings, No. 21, 1974.



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SECTION II – PRIVILEGED COMMUNICATION

## BIOGRAPHICAL SKETCH

*(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)*

| NAME | TITLE | BIRTHDATE (Mo., Day, Yr.) |
|---|---|---|
| Vidic, Branislav, D. S. | Professor of Anatomy | REDACTED |
| **PLACE OF BIRTH (City, State, Country)** | **PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date)** | **SEX** |
| REDACTED | REDACTED | ☐ Male ☐ Female |

EDUCATION *(Begin with baccalaureate training and include postdoctoral)*

| INSTITUTION AND LOCATION | DEGREE | YEAR CONFERRED | SCIENTIFIC FIELD |
|---|---|---|---|
| University of Belgrade, Yugoslavia | D.S. | R | Stomatology |

HONORS

| MAJOR RESEARCH INTEREST | ROLE IN PROPOSED PROJECT |
|---|---|
| Ultrastructure, glandular and pulmonary | Co-Investigator |

RESEARCH SUPPORT *(See instructions)*

Princ. Inv.: "The Effect of Tobacco Smoke on Lung Metabolism." Council for Tobacco Research Inc., U.S.A. #936.340,000 (7/1/74-12/31/76) Effort - 25%.

Co-Inv.: Program Project, "Cellular and Immunological Bases of Pulmonary Disease." HL AI 16748-01 (5/1/74-4/30/79). Effort - 30%.

Princ. Inv.: "The Effect of Pulmonary Blood Flow on Surfactant Synthesis Washington Heart Association #752. $10,350 (1/1/75-12/31/76)

RESEARCH AND/OR PROFESSIONAL EXPERIENCE *(Starting with present position, list training and experience relevant to area of project. List or most representative publications. Do not exceed 3 pages for each individual.)*

Professor of Anatomy, School of Medicine and Dentistry, Georgetown University, Washington, D. C. 1974-present.

Associate Professor Anatomy, School of Medicine and Dentistry, Georgetown University, Washington, D. C. 1971-1974.

Associate Professor of Anatomy, School of Medicine and Dentistry, St. Lou University, St. Louis, Missouri. 1968-1971.

Assistant Professor of Anatomy, School of Medicine and Dentistry, St. Lou University, St. Louis, Missouri. 1965-1968.

First Assistant in Anatomy, School of Medicine, University of Lausanne, Switzerland. 1962-1965.

Visiting Assistant in Anatomy, School of Medicine, University of Basel, Switzerland. Summer, 1961.

Assistant in Anatomy, School of Medicine, University of Novi Sad, Yugoslavia. 1960-1962.

Demonstrator in Anatomy, School of Medicine and Stomatology, University o Belgrade, Yugoslavia. 1954-1958.

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PARTIAL LIST OF PUBLICATIONS

The structure of the palatum osseum and its oral overgrowth. Acta Anatomica 71:94-99, 1968.

The sacral part of the sympathetic trunk in human fetuses and newborn. Archives d'Anatomie, d'Histologie et d'Embryologie 52:35-42, 1969 (W. Wozniak and B. Vidic).

The origin and the course of the communicating branch of the facial nerve to the lesser petrosal nerve in man. Anatomical Record 162:511 516, 1968.

Extreme development of the paranasal sinuses: Report of a case. Anna of Otology, Rhinology and Laryngology 78:1291-1298, 1969.

The communicating branch of the facial nerve to the lesser petrosal nerve in human fetuses and newborns. Archives d'Anatomie, d'Histologi et d'Embryologie 52:371-378, 1969 (B. Vidic and W. Wozniak).

The connections of the intra-osseous segment of the facial nerve in baboon (Papio sp.). Anatomical Record 168:477-490, 1970.

The morphogenesis of the lateral nasal wall in the early prenatal life of man. American Journal of Anatomy 130:121-140, 1971.

The prenatal morphogenesis of the lateral nasal wall in the rat (Mus rattus). Journal of Morphology 133:303-317, 1971.

The variations in height of the palatum osseum as a function of the variations of some other vertical dimensions and angles of the skull. Journal of Dental Research 50:14-17, 1971.

The intermediate root of the trigeminal nerve in the dog (Canis familiaris). Anatomical Record 169:697-703, 1971 (J. Augustine, B. Vidic and P. Young).

Metoda disekcije zdrela. Acta Medica Yugoslavica 24:241-250, 1970.

The histochemical and microscopical differentiation of the respirator glands around the maxillary sinus of the rat. American Journal of Anatomy 132:491-514, 1971 (B. Vidic and H. Greditzer).

The respiratory glandular system in the rat lateral nasal wall in nor and polluted environments. Verhandlungsbericht des Anatomischen Gesellschaft, Anatomische Anzeiger 130:83-85, 1972 (B. Vidic, J. Taylor, M. Rana and B. Bhagat).

Comparative morphology of the communicating branch of the facial nerve Arkhiv Anatomii Gistologii Embriologii (Moscow) 62:17-21, 1972.

The dimensions and shape of the human maxillary sinus in the perinatal period. Acta Anatomica 83:411-415, 1972 (R. Cullen and B. Vidic).

The structure and prenatal morphogenesis of the nasal septum in the ra (Mus rattus). Journal of Morphology 137:131-149, 1972 (B. Vidic, H. Greditzer and W. Litchy).



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SOCIAL SECURITY NUMBER
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The structure of the acinar cell and its relationship to the nerve terminals in the lateral nasal gland of the rat. Archivum Histologicu Japonicum 34:449-461, 1972 (B. Vidic and J. Taylor).

Functional behavior and structural modification of pulpal fibroblasts cultured in vitro. Archives d'Anatomie, d'Histologie et d'Embryologie 55:51-73, 1972 (B. Vidic, R. Chen and J. Taylor).

A reversible damage of the rat upper respiratory tract caused by cigarette smoking. Archives of Otolaryngology (B. Vidic, M. Rana and B. Bhagat) 99:110-113, 1974.

Structure and cytochemistry of the acinar cell in the rat maxillary gland. American Journal of Anatomy 137:102-117, 1973.

Uptake of marker particles by in vitro ventilated and perfused rat lung. American Journal of Anatomy 138:521-527, 1973.

Modifications, some cytochemical properties and transport of intra-lysosomal membranes. American Journal of Anatomy 141:361-373, 1974.

Effect of ventilation on uptake of $^{3}$H-palmitate by isolated, perfused rat lung. Journal of Cell Biology 63:359A, 1974.

BOOKS AND CHAPTERS IN BOOKS

Manual of Human Dissection, St. Louis University Publication, St. Loui Missouri, 1970.

An Atlas of the Anatomy of the Ear, W. B. Saunders Co., Philadelphia, 1971 (B. Vidic and R. O'Rahilly).

Radiological Anatomy of the Nasal and Paranasal Cavities, W. H. Green St. Louis, under consideration (B. Vidic, J. Fries and J. Martin).

An Atlas of the Anatomy of the Larynx, accepted for publication by W. B. Saunders Co., Philadelphia. To be published in 1975 (R. O'Rahilly, B. Vidic and J. Tucker).

Maxillary Sinus, in: Orban's Oral Histology, accepted for publicatio by Mosby Co., St. Louis. To be published in 1975.



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Curriculum Vitae

Jean Rew Wrathall, Ph.D. Born: REDACTED

REDACTED

Education and Professional Experience:

Drew University, Madison, N. J.
University of Utah, Salt Lake City, Utah REDACTED
B.S. cum laude, 1964
Ph.D., 1969. Major: Genetics; Minor: Molecular Biology
Assistant Professor, Biology Department, State University of New York at Geneseo 1969-1970
Instructor of Genetics, Department of Obstetrics & Gynecology, Cornell University Medical College 1970-1972
Postdoctoral Fellow, Damon Runyon Memorial Fund for Cancer Research. Sponsor: Dr. Selma Silagi 1971-1973
Assistant Professor of Genetics, Department of Obstetrics & Gynecology, Cornell University Medical College 1973-1974
Research Associate, Department of Anatomy, Georgetown University School of Medicine, Washington, D. C. 1975-

Publications:


1. Silagi, S. and J.R. Wrathall. 1972. Suppression of tumorigenicity and tyrosinase activity with 5-bromodeoxyuridine. Internat. Cancer Conf. and VIIIth Internat. Pigment Cell Conf. Sydney, Australia, p. 91. (Abstract)

2. Oliver, C., J. Wrathall, S. Silagi and E. Essner. 1972. Suppression of tyrosinase activity and melanosome formation by BrdU in cultured mouse melanoma cells. J. Cell Biol. 55, 194a. (Abstract)

3. Silagi, S., D. Beju, J. Wrathall and E. deHarven. 1972. Tumorigenicity, immunogenicity, and virus production in mouse melanoma cells treated with 5-bromodeoxyuridine. Proc. Nat. Acad. Sci., USA 69, 3443-3447.

4. Wrathall, J.R., C. Oliver, S. Silagi and E. Essner. 1973. Suppression of pigmentation in mouse melanoma cells by 5-bromodeoxyuridine. Effects on tyrosinase activity and melanosome formation. J. Cell Biol. 57, 406-423.

5. Wrathall, J.R. and S. Silagi. 1973. Suppression of pigmentation in mouse melanoma cells by 5-bromodeoxyuridine: Effects on tyrosinase activity. Yale J. Biol. Med. 46, 427. (Abstract)




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R: REDACTED MATERIAL

Curriculum Vitae

Born: REDACTED

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6. Schulman, J.D., J.R. Wrathall, S. Silagi and L. Doores. 1974. Altered amino acid concentrations accompanying suppression of malignancy by 5-bromodeoxyuridine. J. Nat. Cancer Inst. 52, 275-277.

7. Silagi, S. and J.R. Wrathall. 1974. Reversible suppression of differentiation and malignancy in bromodeoxyuridine-treated melanoma cells. In: Makahara, W., T. Ono, T. Sugimura and H. Sugano (Eds.) "Differentiation and Control of Malignancy of Tumor Cells." Univ. of Tokyo Press, Tokyo, pp. 361-385. Review

8. Wrathall, J.R., S. Silagi, R. Balint and E. Newcomb. 1974. Kinetics of suppression of melanoma cell tyrosinase activity and tumorigenicity by 5-bromodeoxyuridine. J. Cell Biol. 63, 379a. (Abstract)

9. Oliver, C., J. Wrathall, S. Silagi and H. Haimes. 1974. Effects of dexamethasone on mouse melanoma cells after long term culture in BrdU. J. Cell Biol. 63, 251a. (Abstract)

10. Wrathall, J.R. and S. Silagi. Suppression of melanoma cell tyrosinase activity after incorporation of 5-bromo-deoxyuridine during one round of DNA synthesis. Proceedings of the IX International Pigment Cell Conference, S. Karger, Basal. In press (Article)

11. Silagi, S., E. Newcomb, J.R. Wrathall and J.K. Christman. Biochemical, immunological and morphological changes correlated with loss of tumorigenicity in 5-bromodeoxyuridine-grown melanoma cells. Proceedings of the IX International Pigment Cell Conference. S. Karger, Basal. In press (article)

12. Wrathall, J.R., E.W. Newcomb, R. Balint, L. Zeitz and S. Silagi. Suppression of melanoma cell tyrosinase activity and tumorigenic after incorporation of bromouracil for one or two cell divisions J. Cell Physiol. In press (article)




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R: REDACTED MATERIAL

CURRICULUM VITAE

GERALD C. GOERINGER

Born : REDACTED

REDACTED

Education and Professional Experience: University of Pennsylvania Philadelphia, Pa. A.B. R (Major: Zoology; Minor: Chemistry

- Hahnemann Medical College Graduate School of Medicine Certificate, R (Isotope Methodology)
- Johns Hopkins University, REDACTED
- Marine Biological Laboratory, Woods Hole, Mass. Certificate, 1957 (Embryology)
- Northwestern University, Evanston, Illinois. United States Public Health Service Predoctoral Fellowship. Ph. D., 1959, (Embryology) Thesis: Modified Development of the Integument of Hypophysectomized Chick Embryos. I. The Epidermis. II. The Feather Germs.
- The Life Insurance Meidcal Research Fund. Postdoctoral Research Fellow, 1959-1962
- Research Associate in Immunochemistry at the Werner-Gren Institute, Stockholm, with Dr. Peter Perlman. 12 months, 1962-1963
- Assistant Professor of Biology, San Diego State College, San Diego, California, 1963-1966
- Assistant Professor of Anatomy, Georgetown University Schools of Medicine and Dentistry, Washington, D.C., 1966-1970
- Associate Professor of Anatomy, Georgetown University, 1970-date

List of Publications:

Some Attempts to Culture Aggregates of Dissociated Tubularia Cells in Glucose Solution. 1957, Marine Biological Laboratory Embryology Abstracts. Supported by Research Facilities Award, M.B.L.

The Effects of Sodium Cyanide and Ethyl Alcohol on the Polarity of of Regenerating Tubularia Stems. 1957, Marine Biological Laboratory Embryology Abstracts.

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R: REDACTED MATERIAL

CURRICULUM VITAE

GERALD C. GOERINGER

Born : REDACTED

Education and Professional Experience: University of Pennsylvania Philadelphia, Pa. A.B. R (Major: Zoology; Minor: Chemistry

- Hahnemann Medical College Graduate School of Medicine Certificate, 1955 (Isotope Methodology)
- Johns Hopkins University, R
- Marine Biological Laboratory, Woods Hole, Mass. Certificate, 1957 (Embryology)
- Northwestern University, Evanston, Illinois. United States Public Health Service Predoctoral Fellowship. Ph. D., 1959, (Embryology) Thesis: Modified Development of the Integument of Hypophysectomized Chick Embryos. I. The Epidermis. II. The Feather Germs.
- The Life Insurance Meidcal Research Fund. Postdoctoral Research Fellow, 1959-1962
- Research Associate in Immunochemistry at the Werner-Gren Institute, Stockholm, with Dr. Peter Perlman. 12 months, 1962-1963
- Assistant Professor of Biology, San Diego State College, San Diego, California, 1963-1966
- Assistant Professor of Anatomy, Georgetown University Schools of Medicine and Dentistry, Washington, D.C., 1966-1970
- Associate Professor of Anatomy, Georgetown University, 1970-date

List of Publications:

Some Attempts to Culture Aggregates of Dissociated Tubularia Cells in Glucose Solution. 1957, Marine Biological Laboratory Embryology Abstracts. Supported by Research Facilities Award, M.B.L.

The Effects of Sodium Cyanide and Ethyl Alcohol on the Polarity of of Regenerating Tubularia Stems. 1957, Marine Biological Laboratory Embryology Abstracts.



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Modified Development of the Integument of Hypophysectomized Chick Embryos. I. The Epidermis. II. The Feather Germs. 1960, Dissertation Abstracts.

Antigens in Microsomes and other Subcellular Fractions of Parenchymal and Reticuloendothelial Cells of Rat Liver. Agar Diffusion Studies. 1964, in H. Peeters (Ed.), Protides of the Biological Fluids, 12th Colloquium, pp. 232-235. Elsevier, Amsterdam. (With P. Perlman and U. Lundkvist)

Parenchymal and Reticuloendothelial Cells of Rat Liver. Fluorescent Antibody Staining. 1964, in H. Peeters (Ed.), Protides of the Biological Fluids, 12th Colloquium, pp. 236- 241. Elsevier, Amsterdam. (With U. Lundkvist and P. Perlman)

Immunochemical Characterization of Parenchymal and Reticuloendothelial Cells of Rat Liver. 1966, Exp. Molec. Pathol. 5: 427-442. (With U. Lundkvist and P. Perlman)

Development of the Epidermis in Hypophysioprivic Chick Embryos. 1968, Anat. Rec. 160: 354.

Some Changes in Chorionic Epithelium undergoing Keratogenous Metaplasia. (With P.L. Morwood and T.M. Walker), 1969, Anat Rec. 163: 232-233.

Appearance of Enzyme Activity During Metaplastic Changes of the Chick Embryo Chorioallantoic Membrane. (With P.L. Morwood and T.M. Walker) 1970, Proc. Soc. Exp. Biol. Med. 134: 539-541.

Disc Electrophoresis of the Rat Oviduct and its Epithelium. 1971, Anat. Rec. 169: 325. (With C.W. Lynn).

Development of Epidermal Enzymes in the Hypophysectomized Chick Embryo Anat. Rec. (Accepted for publication) With P.L. Morwood.

The Role of the Pituitary in Development of Hydrolytic Enzymes in Chick Embryo Epidermis Transplanted to Chorioallantoic Membrane (With P.L. Morwood). In preparation.



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R: REDACTED MATERIAL

Curriculum Vitae

Max Robinowitz, M.D.

| | |
|---|---|
| Date of Birth: | REDACTED |
| Education: | Georgetown University, Washington, D.C., B.S. Honors in Biology. R |
| | Georgetown University, Washington, D.C., M.D. Cum Laude R |
| Training: | Barnes Hospital, St. Louis, Missouri, Straight Medicine Internship 1961-62; Medical Resident 1962-63. |
| | Mount Sinai Hospital, New York, New York, Pathology Resident 1963-67; Chief Resident in Pathology, 1965-67. |
| Military Service: | U.S. Army, Major MC, USAR, 1967-69; Walter Reed General Hospital, Washington, D.C. Assistant to Chief of Anatomic Pathology, 1967-68; Assistant Chief of Clinical Pathology, 1968-69. |
| Board Certification: | Anatomic and Clinical Pathology, 1967. |
| Experience: | Armed Forces Institute of Pathology, Washington, D.C., Assistant Pathologist, Obstetric, Gynecologic, and Breast Branch, 1969-70. |
| | Mount Sinai Hospital, Miami Beach, Florida, Associate Pathologist, 1970-72. |
| | Director, Radioimmunoassay Section, Georgetown University Hospital, 1972-1975. |
| | Staff Pathologist, Georgetown University Hospital (Anatomic and Clinical Pathology) 1972- |
| University Appointments: | Washington University School of Medicine, St. Louis, Missouri, Assistant in Medicine, 1962-63. |
| | Mount Sinai School of Medicine, New York, New York, Instructor in Pathology, 1965-67. |
| | University of Miami School of Medicine, Miami, Fla. Assistant Professor of Pathology, 1970-72. |
| | Georgetown University School of Medicine, Assistant Professor of Pathology 1972- |

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Committees: Interviewer for Georgetown University Medical School Admissions Committee, 1972-

Member, Georgetown University Medical Library Committee, 1972-

Member, Georgetown University Medical Center Laboratory Committee, 1972-

Teaching Appointments: Georgetown University School of Medicine:

Faculty member, Sophomore Pathology Course for Medical and Dental Students

Faculty member, Introduction to Medicine Elective for Freshman students (weekly seminar)

Residency training in Anatomic and Clinical Pathology

Interdepartmental conferences with Medicine, Surgery and Pediatrics and Radiology.

Preceptor, Freshman Student Elective

Licensure: Florida, District of Columbia, Maryland
Certification, National Board of Medical Examiners

Honors: Alpha Omega Alpha:
Gold Medal in Medicine, Georgetown University, 1961.

Professional Societies:

REDACTED

REDACTED

Publications: Rivera, R.A., McAllister, H.A., and Robinowitz, M., Fulminant hepatic necrosis following halothane anethesia, Med. Ann. Dist. Columbia, 39: 371-373, 1970.

Norris, H.J., and Robinowitz, M., Ovarian adenocarcinoma of mesonephric type, Cancer 28: 1074-1079, 1971.

Brief Report: Robinowitz, M., Robinson, D.M. and Oppleman, P., Very Low Magnification Microscopic Technic, ASCP Summary Report 9:11, 1972.

Abstract: Robinowitz, M., Mathew, J., Eckelman, W., and Harbert, J.C., Fatal reactions following $^{99m}$Tc- ferrous hydroxide lung scan J. Nucl. Med. 14:445-446, 1973

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# CURRICULUM VITAE

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Jewish Hospital, Cincinnati, Ohio
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Research Associate, Physiology & Biophysics 1974-present time

National Board Status: FLEX - December, 1974

Medical Specialty Board Status: Diplomate American Board of Internal Medicine, 1972

Organizations: REDACTED

Publications:

1 - Da Silva, A. M. T., and Hamosh, P.: Effect of smoking a single cigarette on the "Small Airways". J. Appl. Physiol. 34:361, 1973.

2- Hamosh, P., and Da Silva, A. M. T.: Supine hypoxemia and erythrocytosis due to airway closure at low lung volumes. Am. J. Med. 55:80, 1973.

3- Hamosh, P., and Da Silva, A. M. T. : Air bolus method compared to single breath method for determination of closing volume. Am. Rev. Resp. Dis. 110:518, 1974.

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|---|---|---|---|
| Smoking and the "small airways" P.I. Paul Hamosh | Council for Tobacco Research # 878 | 81,000 | 1/1/73-12/31/75 |
| Smoking and lung metabolism P.I. B. Vidic | CTR #936 | 140,000 | 1/1/74-12/31/76 |
| The Effect of Pulmonary Blood Flow on Surfactant Synthsis P.I. B. Vidic | Washington Heart Association | 10,350 | 1/1/74-12/31/75 |
| Estrogen in Human Adipose Tissue Lipoprotein Lipase P.I. M. Hamosh | Washington Heart Association | 10,350 | 1/1/74-12/31/75 |
| The Role of Chylomicrons in Atherosclerosis P.I. P. Hamosh | Washington Heart Association | 11,500 | 7/1/75-6/30/76 |
| Cellular and Immunological Bases of Pulmonary Disease P.I. Joseph Bellanti | NHLI (HL 16748) | 1,250,000 | 5/1/74-4/30/79 |

PENDING OR PLANNED

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|---|---|---|---|
| Blood Triglyceride and Lipase in the Lung P.I. M. Hamosh (submitted) | National Heart and Lung Institute | 140,540 | 1/1/76-12/31/78 |

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator:

Typed Name: Paul Hamosh M.D.

Signature: [signature] Date: 6/30

Telephone: Area Code REDACTED Number Extension

Responsible officer of institution:

Typed Name: Sam A. Kimble

Title: Administrator of Sponsored Programs

Signature: [signature] Date: 7/7/75

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Checks payable to:

Georgetown University

Mailing address for checks: Sam A. Kimble
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APPENDICES

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19.4 AN AUTOMATIC QUANTITATIVE ESTIMATION OF EMPHYSEMA

Ungerleider, J. A.* and Hamosh, P.**

We have developed an automatic method for transmitting a representation of a section of lung tissue into a computer and quantifying emphysematous destruction. Emphysema by definition is the distention and disruption of air spaces within the lung, with reduction of the internal surface area (ISA). Due to the inhomogeneous distribution of emphysematous lesions in the lung, the estimate of ISA is not sufficient for evaluation of the damage. An automated method for measuring ISA by pattern recognition techniques has been described by Levine et. al. (IEEE, BME 17:254-261, 1970). Our method calculates in addition mean volume, the frequency of disruption, the thickness of the remaining septal structures, and the relative distribution of tissue to air space.

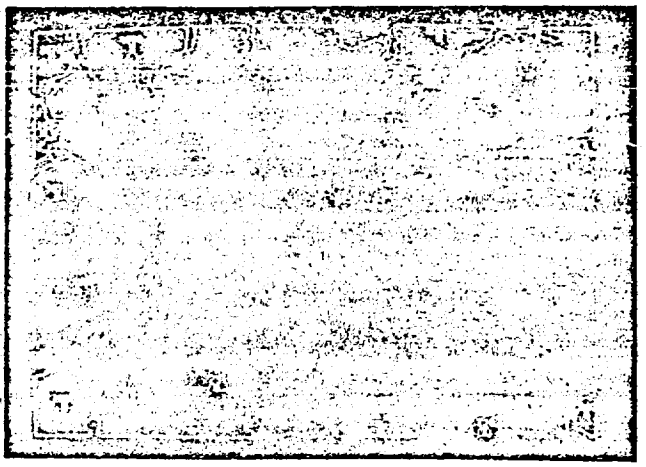
After pressure fixation with 10% buffered formaldehyde and embedding in paraffin, the lungs are sliced into 4 to 8μ sections and stained with hematoxylin and eosin. Sections are then mounted on glass slides which serve as input to the SPIDAC system. This is an automatic optical-microscope scanner for on-line input of pictorial data from a glass slide directly into the core memory of a digital computer. The SPIDAC microscope stage is directly under computer control and capable of locating each field of interest to within 1.25μ maintaining continuous automatic focus. Once each field has been positioned, it is digitized to a raster of 700 x 500 points and 16 gray levels and is transmitted directly to the computer. It is upon this stored image that the analysis programs operate.

For each image there is a certain gray level cut-off value which is used to distinguish the points of the image occupied by tissue from those occupied by air. Once this value is set the separation between these areas is easily made. First, a scan is made of the image raster, counting the number of points above the cut-off value to determine the area occupied by tissue and the number of points at or below the cut-off value for the area occupied by air. Through a method of linear integration an estimate of the internal surface area of the lung is made. This entails noting and keeping track of the gray level values of the points along randomly distributed lines placed upon the stored image. Based upon the cut-off value, it is determined if the point rests on tissue or air and when the line crosses the boundary between the two. The results of imposing several such lines on each image is then used to produce a statistical estimate of the internal surface area.

To investigate the structure of the septal walls a scan is made of the image, horizontal row by horizontal row. When a point with a stored value greater than the cut-off is encountered, it is treated as a boundary point of a septal wall. From here the entire boundary of the septal wall is traced by continuously locating the neighbor of the previous point which borders on points below the cut-off value. As the boundary is being traced, conceptual, programmed "probes" are sent perpendicular to it toward the interior of the wall to determine its width. Secondly, the curvature of the walls is calculated at each point based on the boundary list and from this the number of free ending septae is determined.

A similar scan is made of the image for the detection of objects based on boundary points at or below the cut-off gray level value. This allows the bounding of all areas on the image occupied by air. This boundary list is then used to describe the shape, size, and area of each air-filled region.

Analysis of this information gives in addition to quantitative assessment of the extent of emphysema, data on the distribution and pattern of tissue disruption. This approach will provide a tool to study stress distribution in the lung. Whereas manual methods can be developed, this approach is superior because of the elimination of observer bias and increased speed.



A computer display illustrating the boundary and area of each air space.

This work was supported by grants GM-15192 and RR-05681 from the National Institutes of Health, PHS, DHEW, to the National Biomedical Research Foundation; and contract V688P-160 from the Veterans Administration Hospital to Georgetown University.

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PROCEEDING OF SECOND SYMPOSIUM
ON
SHARING OF COMPUTER PROGRAMS AND TECHNOLOGY IN NUCLEAR MEDICINE

Oak Ridge, Tennessee, April 21-22, 1972

P. HAMOSH M.D.

## Abstract

# Instrumentation for Pattern Recognition and Associated Software

by

Robert S. Ledley and Louis S. Rotolo

In this paper we describe the biomedical pattern recognition system of the National Biomedical Research Foundation. The hardware portion of the system consists of a high-resolution flying-spot scanner (FIDAC), a vidicon scanning system with associated silicon video memory system (VIDIAC), a mechanical scanner of very great resolution (DRIDAC), a microscope scanning system (SPIDAC), and an interactive communication system (MACDAC). The associated software systems used in the operation of the above hardware systems and in the automatic pattern recognition consist of FIDACSYS, which supervises input of the image into the computer from the scanning devices and accomplishes overall picture manipulation, and the SYNTAXSYS and BUGSYS pattern-recognition languages developed at the Foundation. Finally, a system (DOCS) is described which is used for on-line computer-console interaction with disk memories for the evaluation and display of large masses of data.

April 21, 1972

National Biomedical Research Foundation
Georgetown University Medical Center
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## Instrumentation for Pattern Recognition and Associated Software*

The pattern recognition system of the National Biomedical Research Foundation consists of a number of scanners, display consoles, and interactive devices. The laboratory is centered around an IBM 360/44 computer (owned by the Foundation), which is used on a "dedicated" basis. The figures show the organization of the specialized pattern-recognition equipment of the laboratory (see Figs. 1, 2, and 3).

All of the equipment (except the IBM 360/44 computer) is of our own design and construction; either it was not available from commerical electronic manufacturers or its requirements exceeded the specifications of available apparatus. All of the scanners and interactive displays are integrated into a single configuration through the Pattern Recognition Control System. The SPIDAC (Specimen Input to Digital Automatic Computer) consists of an automated microscope with a motorized stage which is under computer control in both the x and y directions; it includes an instantaneous automatic-focus device. The VIDIAC (Vidicon Input to Automatic Computer) scans the image at high resolution and stores it temporarily on the Silicon Video Memory System (the heart of which is a new image-converter tube). The stored image can then be read directly into the computer's memory (through the Pattern Recognition Control System), or displayed on the grey-level TV monitor or the MACDAC interactive monitor. The MACDAC (Man Communication with Digital Automatic Computer) enables interactive communication with the computer by means of a "joy stick" cursor. The FIDAC (Film Input to Digital Automatic Computer) is a flying-spot scanner for scanning film transparencies at very high resolution; it is also used to make a print or film negative under the control of the computer, with high spatial and grey-level resolutions. The DRIDAC (Drum Input to Digital Automatic Computer) is a mechanical scanner of very great resolution in which a picture or X ray is wrapped around a drum and scanned by several photocells moving along a worm gear as the drum rotates.

All of the object scanning units (namely the VIDIAC, FIDAC, and DRIDAC) have systems that image and digitize the object picture; the digitized information can be sent to the core memory of the computer. Also, each of the scanned images can be stored on the Silicon Video Memory, in either video (analog) or digital form (i.e., a particular grey level or levels); many options can be selected by the user. From the Silicon Video Memory, the stored image can be displayed with grey levels on the TV monitor. The scanned images can also be stored on the MACDAC monitor (which is a storage tube). Then an interactive-graphics phase of a program can be performed in which the operator can present to the computer the x and y coordinates of a point, and

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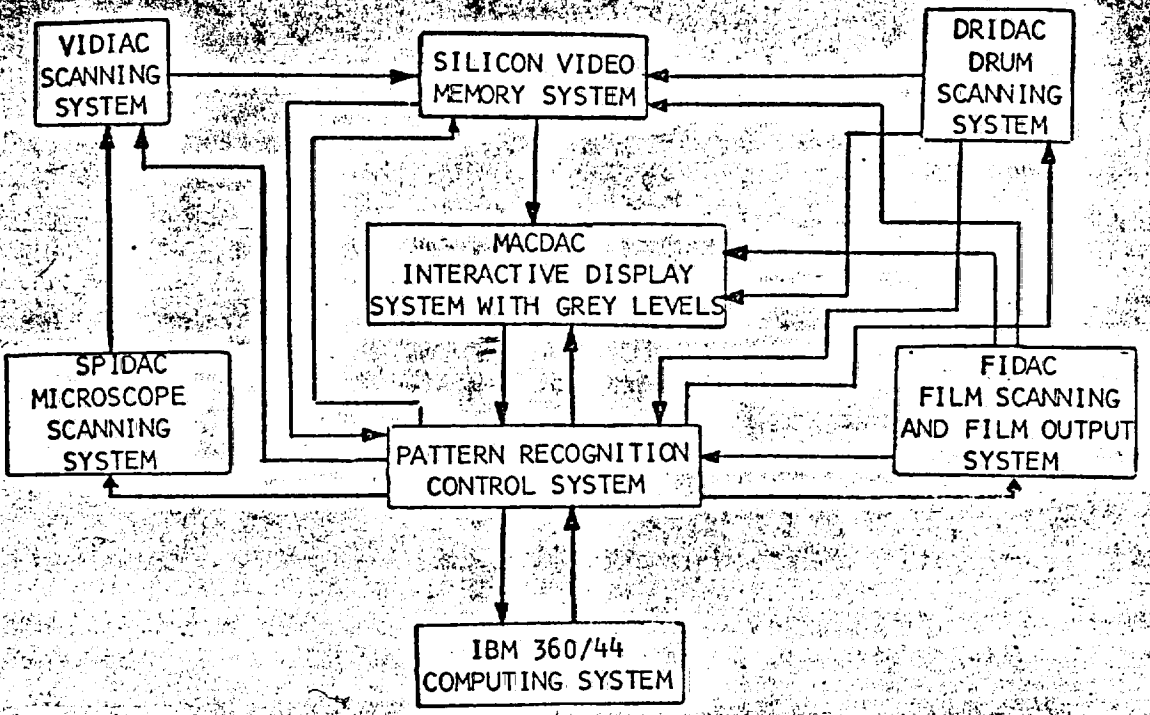


Fig. 1. National Biomedical Research Foundation Automatic Pattern Recognition and Computing Laboratory.

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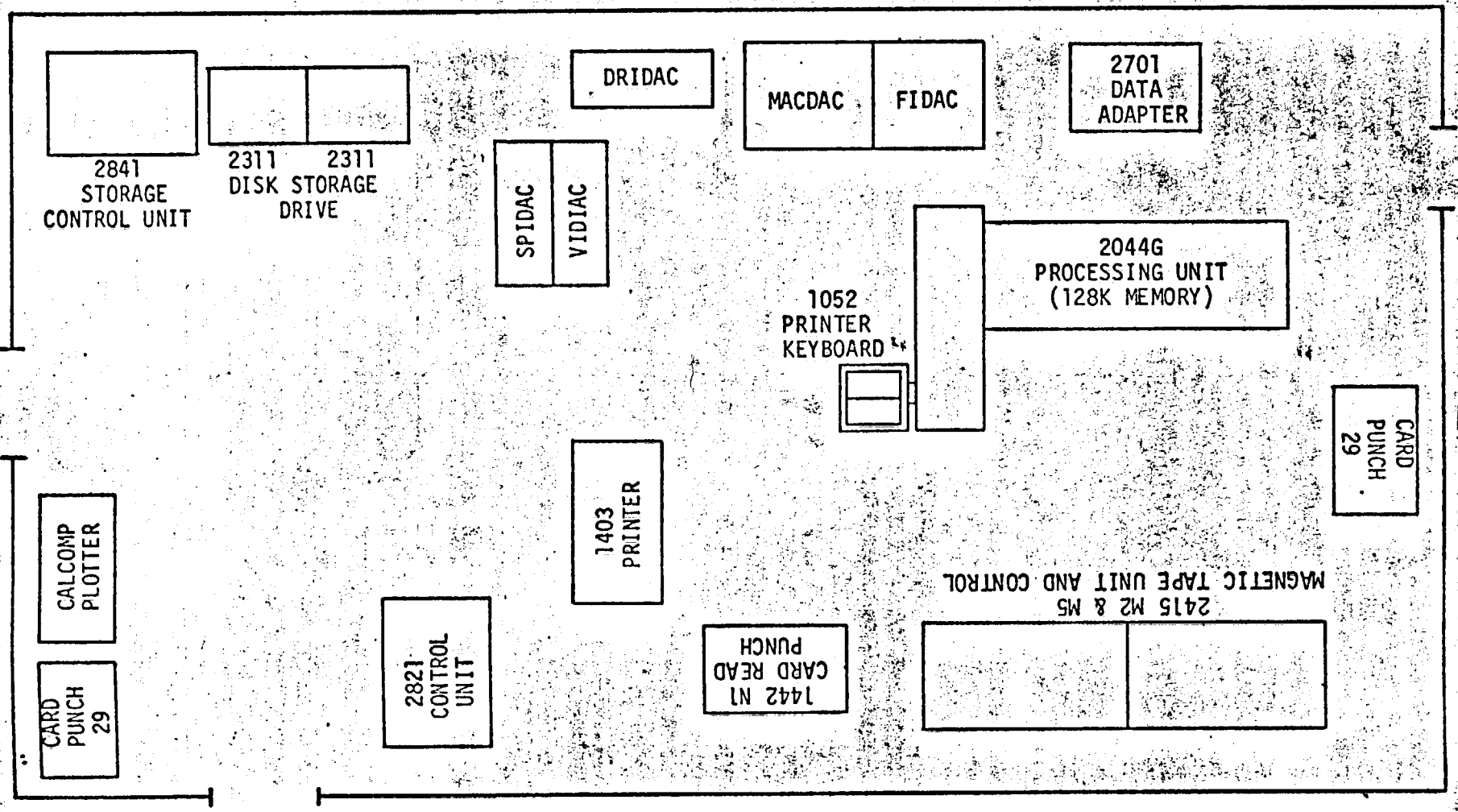


NBR System 360/44

Fig. 2. Layout of the National Biomedical Research Foundation Pattern Recognition Laboratory and Computer Center.

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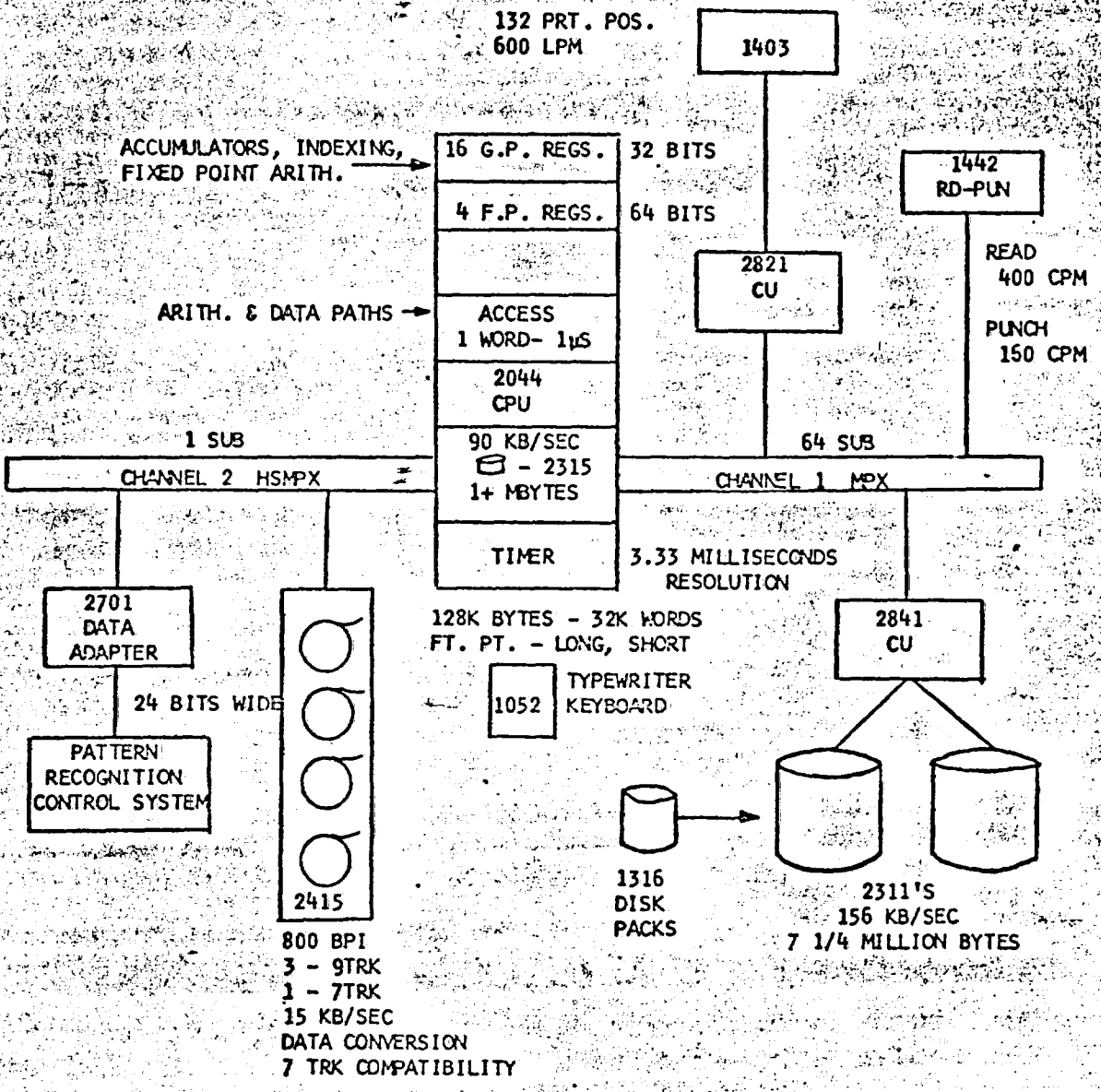


Fig. 3. National Biomedical Research Foundation 360/44 Configuration.

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some coded information concerning the nature of the point. Finally, the computer itself can draw a picture on the MACDAC monitor, or on the TV monitor via the Silicon Video Memory System (or on a hard-copy printer via the Silicon Video Memory System). Thus the computer can present a MACDAC display, a TV monitor grey-level display, a hard-copy grey-level display, and a high-resolution grey-level display on film by means of the FIDAC.

The specialized pattern-recognition equipment of the laboratory includes:

(a) FIDAC (Film Input to Digital Automatic Computer) Mod II. The FIDAC is a high-speed high-resolution flying-spot scanner developed and built at the Foundation. It has a maximum capability of scanning a raster of 2000 x 2000, or 4,000,000 sample points within four seconds (i.e., at the rate of one microsecond per sample spot), with up to 16 grey levels per point. The number of sampled spots in the raster is adjustable, as is the number of grey levels. For instance, for the analysis of chromosomes by classical (nonbanding) methods, a nominal 700 x 500, or 350,000 sample-spot, raster is most efficacious. The analysis of fluorescent chromosomes is always accomplished from film, for the fluorescence fades as it is exposed to ultraviolet light. Thus a photomicrographic film is made in which the fluorescence from all the chromosomes can be recorded at the same time. (See Fig. 4.)

The FIDAC has two modes of operation, as follows: (1) A complete picture mode. In this mode the raster's size is nominally 700 x 500, that is, the picture is sampled at 350,000 spots, and each point can be sampled as black-and-white binary, i.e., one bit per spot, or up to 16 grey levels, i.e., four bits per spot. This raster size is used for the examination of biomedical photomicrographs which were obtained from an optical microscope, since this sampling rate exceeds the resolution of the optical microscope. (2) Control word mode. In this mode of operation a resolution of 2,048 spots across each of 1,365 rows (3 x 2 aspect ratio) with a maximum of 16 intensity grey levels can be obtained. This is accomplished by allowing the computer program to select all or any portion of the picture to be scanned with a corresponding sampling density such that the memory space allocated to the picture would not be overrun.

The specifications of FIDAC are as follows: A 35-mm still-frame film transport is used which can take up to 250 frames. The film frames can be advanced automatically under computer control. The logical circuitry utilizes integrated circuits soldered to printed circuit cards that are plugged into racks for ease of maintenance. The system is of our own design, completely synchronous, and can be clocked at any frequency up to 10 megahertz. The circuitry is geared to an easily adjustable master clock. Thus the clock speed can be increased or decreased, depending upon the characteristics of the computer to be used. The circuitry matching FIDAC to the computer consists of several replaceable cards. These FIDAC-computer interfacing cards are the only circuitry that would have to be changed in shifting from one computer system to another. The spot size is 1.5 mils at one foot-Lambert light output (measured on the face of the CRT). At the 1.5 mil spot size and at 2,000 spots per x-sweep of the raster, there is no overlap of spots because the x-scan raster is 3 inches wide. The rise time of the photomultiplier is 2.3 nanoseconds, the video-amplifier bandpass is 10 MHz (megacycles), and the phosphor persistence is 100 nanoseconds. Thus one sample spot per microsecond clearly presents non-overlapping spots. Dynamic focusing is incorporated in FIDAC. This presents an almost constant spot size from the center to the sides of the CRT. The sampling rate used is 1 microsecond per spot. This means that the four-bit 16-grey-level analog-to-digital conversion takes place completely within this time period. One frame is scanned in about 0.35 seconds for complete picture mode (700 x 500 raster), and

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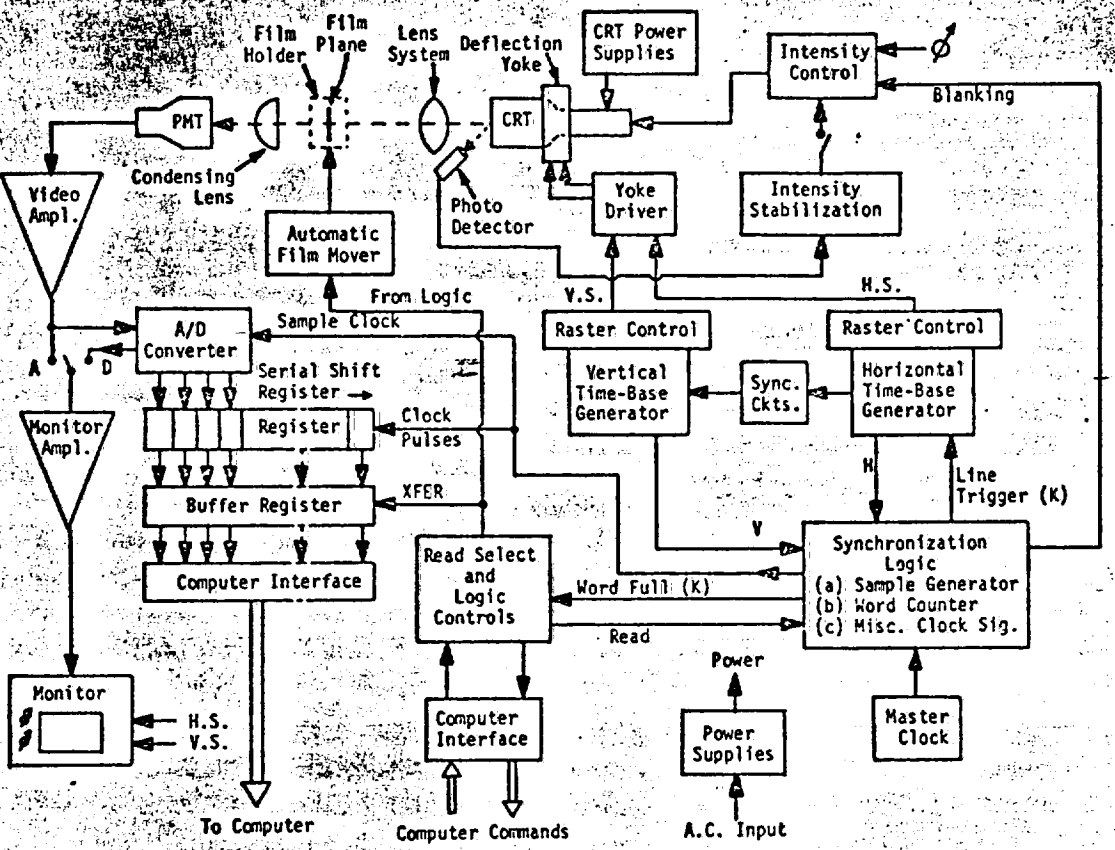


Fig. 4. . The Basic FIDAC System.

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three seconds for a 2,000 x 1,365 raster. Jitter is related to the synchronization of the triggering of each successive line of the raster. The jitter of the FIDAC is less than 1 spot. Antipincushioning coils are included in both the x and y directions. By aspect ratio is meant the ratio of the distance between adjacent spots in the horizontal and vertical directions. It is usually desired to keep this at unity in order that no vertical or horizontal distortion occurs. On FIDAC the aspect-ratio is retained when the raster size is changed. The FIDAC can be used with 2 through 16 grey levels for each spot. Each grey level can be set within 0.1%. Shading refers to the correction of the grey-level variation across the area of the picture that arises from the CRT, photomultiplier, and optical systems. For the FIDAC, the shading generator can be adjusted so that for a blank frame an almost uniform response is obtained across the whole frame. If successive scans are made on the same picture frame, then the starting point, or very first spot, on such successive frames will be at the same place in the picture frame to within one spot diameter. The raster size can vary from line to line in length of line or in the rate of scanning within the line. The stability of such possible errors is within one spot diameter. The cutoff voltage of each level detector is stable within .01%. The drift in the output of the video amplifier has been stabilized to maintain a constant output for a given input signal. A feedback loop adjusts the average integrated CRT-spot intensity of the frame to a constant value.

(b) VIDIAC (Vidicon Input to Automatic Computer). The VIDIAC device was also designed and constructed by our electronic laboratory either for scanning through a microscope or for scanning large pictures or X rays. It consists of a high resolution vidicon system that scans at standard TV rates (i.e., 1/30 sec/frame), coupled to a Silicon Video Memory System. The standard vidicon scan rate is too fast for most analog-to-digital conversion systems and for data transmission to a digital computer. Hence the Silicon Video Memory System acts as an interface between the vidicon and the computer. The capabilities of the VIDIAC are as follows. The image scanned by the vidicon can be displayed on a TV monitor for viewing and for optimizing such parameters as focus, intensity, contrast, etc. The image scanned by the vidicon can be written onto the Silicon Video Memory System. The electronic image as stored in this silicon memory can in turn be internally scanned and displayed on either the TV monitor or on the storage tube of the MACDAC. A zoom control on the silicon memory and a bias control on the console of the MACDAC can be used to display any portion of the electronic image or to read into the computer any portion of the image. This zoom feature is also under computer control. The standard vidicon scan rate of 1/30 sec. per frame is equivalent to about a 30 megahertz spot sampling rate; this fast scan is the rate from the vidicon to the silicon memory tube and from the silicon memory to the TV monitor. The equivalent spot sampling rate from the silicon memory to the computer or to the MACDAC, or of writing onto the silicon memory from the computer, called the slow scan, is a maximum of a megahertz, but frequently 1/3 megahertz is used. Switching the flow of information between the vidicon, TV monitor, MACDAC, and computer, together with the appropriate scan rates, is under both manual and computer control. (See Fig. 5.)

(c) SPIDAC (Specimen Input to Digital Automatic Computer). The SPIDAC is an instrument for scanning directly through a microscope, digitizing the resulting grey-level pattern, and placing the digitized image directly into the computer's memory. The system is able to automatically manipulate the microscope slide in both the x and y directions, record the coordinates of objects of interest, automatically focus the image instantaneously, and monitor results, all under computer control. Eliminating the photographic

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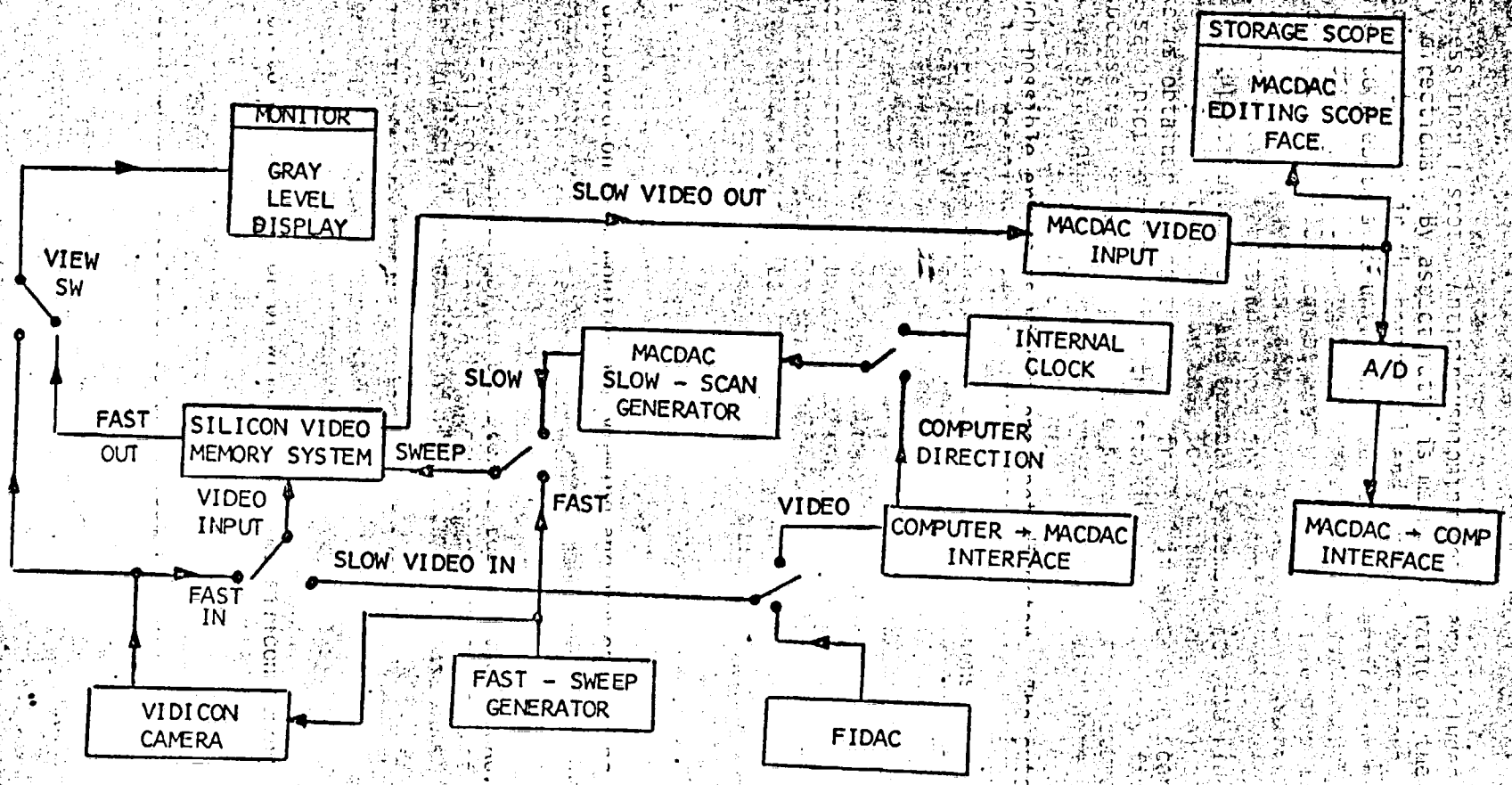


Fig. 5. Block Diagram of VIDIAC System.

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step has the advantage of saving time and effort. Also, in a number of instances it is possible that such direct digitization may bring more sensitivity to grey-level discrimination and can eliminate certain possible grey-level distortions which can occur when using film as an intermediate step. (See Fig. 6.)

One of the main functions of the SPIDAC is to locate specimens of interest on the glass slide by automatic means, provided that an object of interest can be specified utilizing pictorial parameters. The SPIDAC system performs its operation in two phases. During the first phase a coarse scan (40x objective) is made to determine where the objects of interest are located. During the second phase a final and more accurate determination (at 100x objective) is made of whether or not the object located in the first stage is of sufficient interest to be automatically analyzed, and if so then its image is scanned by the VIDIAC and digitized and read into the computer. The pattern-recognition programs in the computer are utilized to compute the desired quantitative information from the pictorial data. The 40x and 100x objectives are parfocal, and a filter is introduced when the 100x is in place so that no adjustment of the vidicon is necessary when going from 40x to 100x objective.

In particular, SPIDAC scans a 1 cm x 1 cm field on the glass slide. At 40x objective, the frame size is 200μ x 260μ, giving about 40 strips of 50 frames each (or 2,000 frames) over the 1 $cm^2$ area field. Each of the x and y stepping motors is run at a rate of 650 steps/second, with 200 steps/revolution, moving the stage 500μ/revolution. This amounts to 2.50μ/step, meaning that we can locate any point in the field to the nearest 1.25μ. The rate of movement is (650 x 500) ÷ 200 = 1,625μ/second, or 10,000 ÷ 1,625 or about 6 seconds/strip, or finally 6 x 40 = 240 seconds for the 40 strips of the field. To move from strip to strip will take a total of 6 seconds across the field, so the total scan time limitation due to the motors themselves is 246 seconds or 4 minutes and 6 seconds. In addition, each field is examined (in the first phase as described above) for a chromosome spread, and this takes (1/30) second per frame, or 2,000 x (1/30) = 66.6 seconds or 1 minute and 6.6 seconds. Thus the total time that SPIDAC takes to search a 1 cm x 1 cm field area for chromosome spreads is 5 minutes and 12.6 seconds.

(d) MACDAC (MAn Communication with Digital Automatic Computer). The MACDAC is an interactive-graphics device designed and constructed by us for communicating with the computer. Automatic chromosome analysis involves a limited but essential manual "editing" step which is accomplished by means of the MACDAC. After the computer console is properly set up, the control of the complete chromosome analysis is carried out by the operator at the MACDAC console. The expanded MACDAC will include a hard-copy grey level picture printer. Two monitors are available to the MACDAC. The first is a Tektronix storage tube capable of only black and white (actually green) display; the second is a TV monitor capable of grey level display. The interaction is carried out by means of a joy stick controlled cursor. For any position of the cursor, its 10-bit x and 10-bit y coordinates can be read into the computer, together with a four-bit code set by the operator, on a priority interrupt basis. This four-bit code identifies for the computer the meaning of the x and y coordinates. The interrupt capability allows the relatively slow interactive editing process to be carried out at the same time as the computer is executing an analysis program, on an overlapping basis. (See Fig. 7.)

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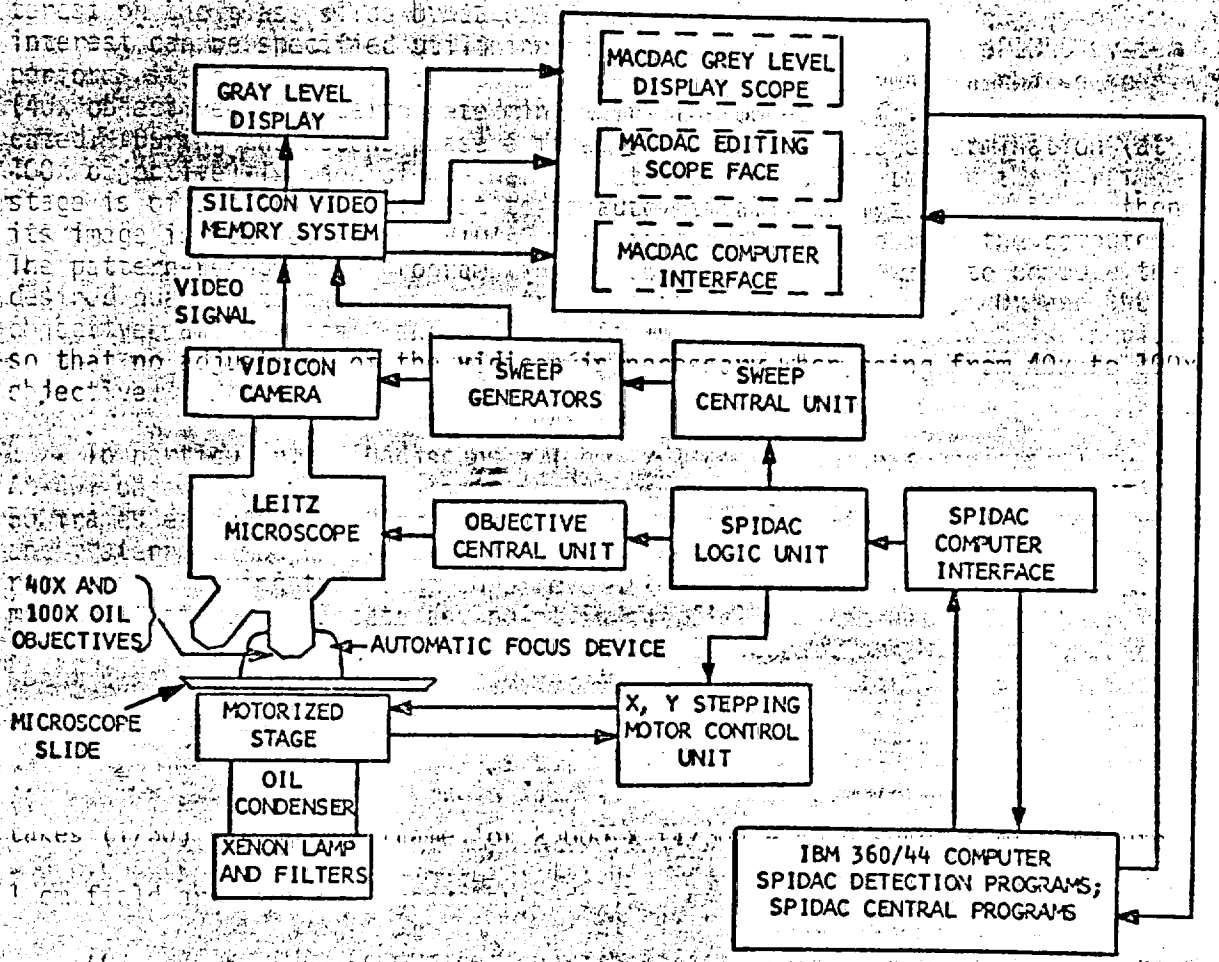


Fig. 6. Block Diagram of SPIDAC system (including the associated parts of MACDAC and COMPUTER hardware and software).

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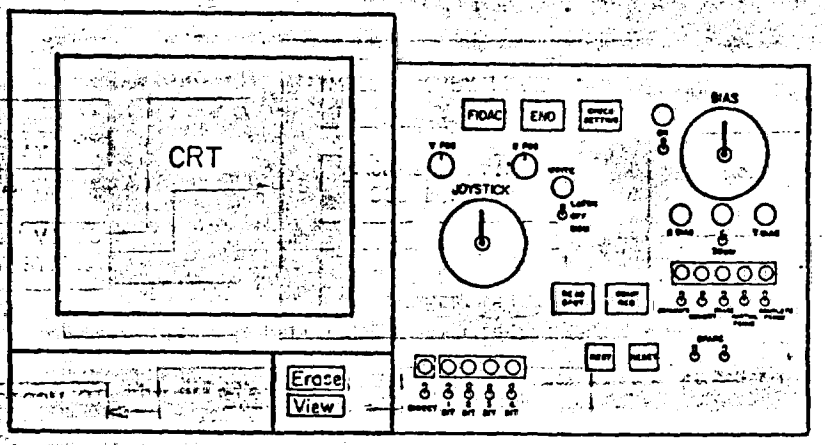
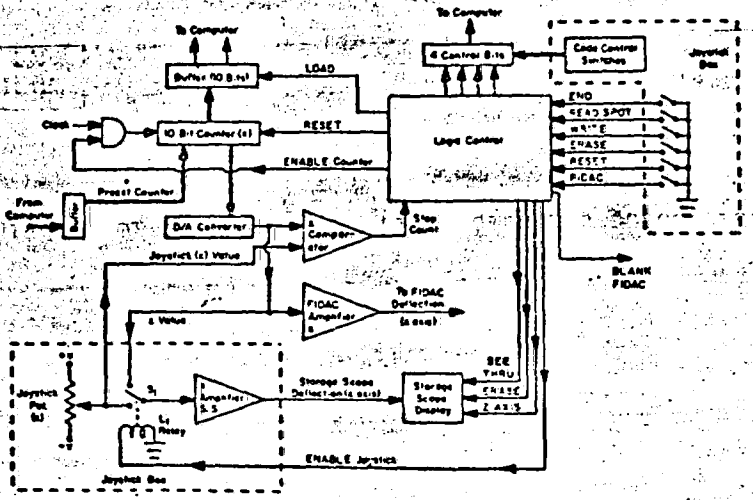



Fig. 7. (a) MACDAC Console: At left is the display tube; at right, the control panel. The lower left (main) joystick controls the cursor; the upper right (bias) joystick controls the zoom feature. The line of five switches below the bias joystick controls the four-bit code which is read in with the coordinates when the "Read Spot" button is pushed. Other, nonstandard codes can be set by means of the five switches below the main joystick.




(b) Some Details of the MACDAC Design.

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The MACDAC has two joy sticks, one for normal interaction, and the second for zoom control. The zoom control enables any area of the display to be enlarged so that detail can be more easily observed. The MACDAC console includes switches to control the scan of the FIDAC or VIDIAC, to tell the computer that the interactive procedure has been completed, to direct the computer to display a picture on the monitors, to tell the computer to enable the use of the joy sticks, and so forth. Either the analog video signal or the digitized signal can be displayed on the MACDAC. For the digital signal, any particular grey level can be seen, or any grey level above a certain cutoff level can be displayed, and so forth.

(e) DRIDAC (DRum Input to Digital Automatic Computer). Many pictures of biomedical importance are produced in relatively large sizes, both as transparencies and black and white prints. Some examples are large chest X-ray plates, X rays of large bones, X-ray crystallographic plates, angiograms, graphs such as electroencephalographs and electrocardiographs, and many others. The DRIDAC device was designed and built at the National Biomedical Research Foundation to allow such pictures to be directly read in to a digital computer. (See Fig. 8.)

DRIDAC is a high-speed, high-resolution, on-line drum scanner which can scan photographs (X ray, transparency, or print) of any size up to sixteen inches by twelve inches of useful area. The resolution may be varied to as fine as 0.004 inches. This allows it to sample 4,000 x 3,000 spots, for a total of 12 million spots, from a large picture. Each spot can be digitized as required into one bit (black and white) or up to six bits (sixty-four grey levels per spot). An automatic background detection and compensation feature when activated affords a much greater dynamic range and in effect increases the number of grey levels. Another innovation in our drum scanner is the utilization of two read stations (see figures) thereby reducing the total scan time to one-half the time required by a single read station.

In consideration of the large quantity of data which can be obtained from a single picture, we have designed the DRIDAC device to read the pictorial data into the core memory of our IBM 360/44 computer at a maximum rate of 252,000 bits per second and simultaneously transfer the accumulating data from the core into the IBM 2311 disk drive pack (see Fig. 9). Since a 2311 disk pack will store approximately 54 million bits, this requires a slight reduction in grey-scale resolution if we are to read in a full 4,000 x 3,000 spot picture.

The DRIDAC's drum speed is 420 rpm and its sampling rate is approximately 35 microseconds per spot. Thus to read in an entire picture requires approximately five minutes. The DRIDAC's high speed in getting a large quantity of data onto the disk saves valuable computer time. Once the data is on the disk, the program can select any desired portion of the picture at disk speed. It would be inappropriate to process data directly from the drum scanner, since backtracking or moving to distant spots under the control of the computer is slow and would therefore waste computer time.

Of no less importance than the hardware (or computer instrumentation) is the associated software (or computer programming) systems that we have developed and are available for use in this work. Since the programming systems or languages are extremely extensive and complex, we can only give the "flavor" of the languages in the following discussions. These software systems include:

The specialized pattern-recognition software of the laboratory includes:

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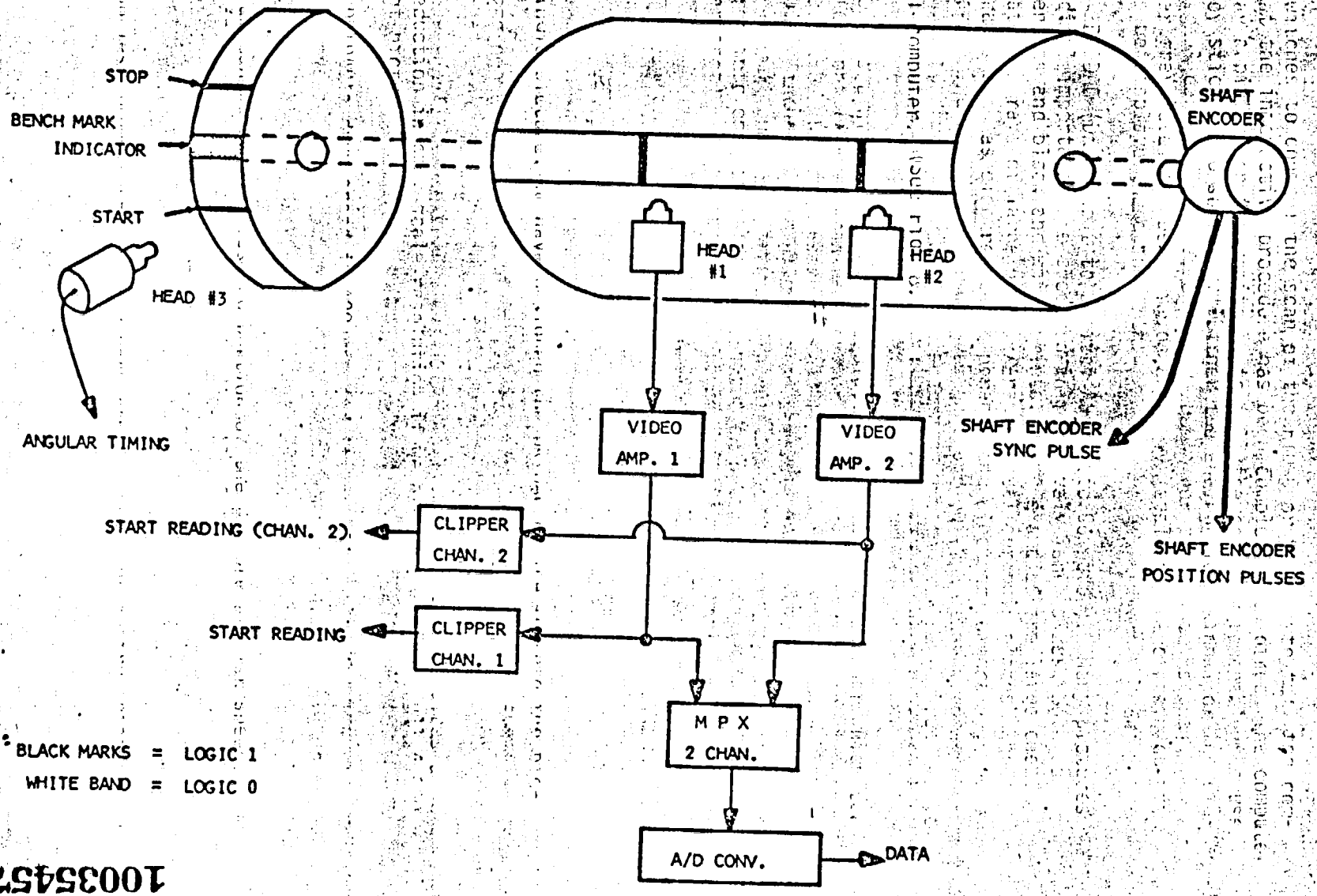


Fig. 8. DRIDAC System.



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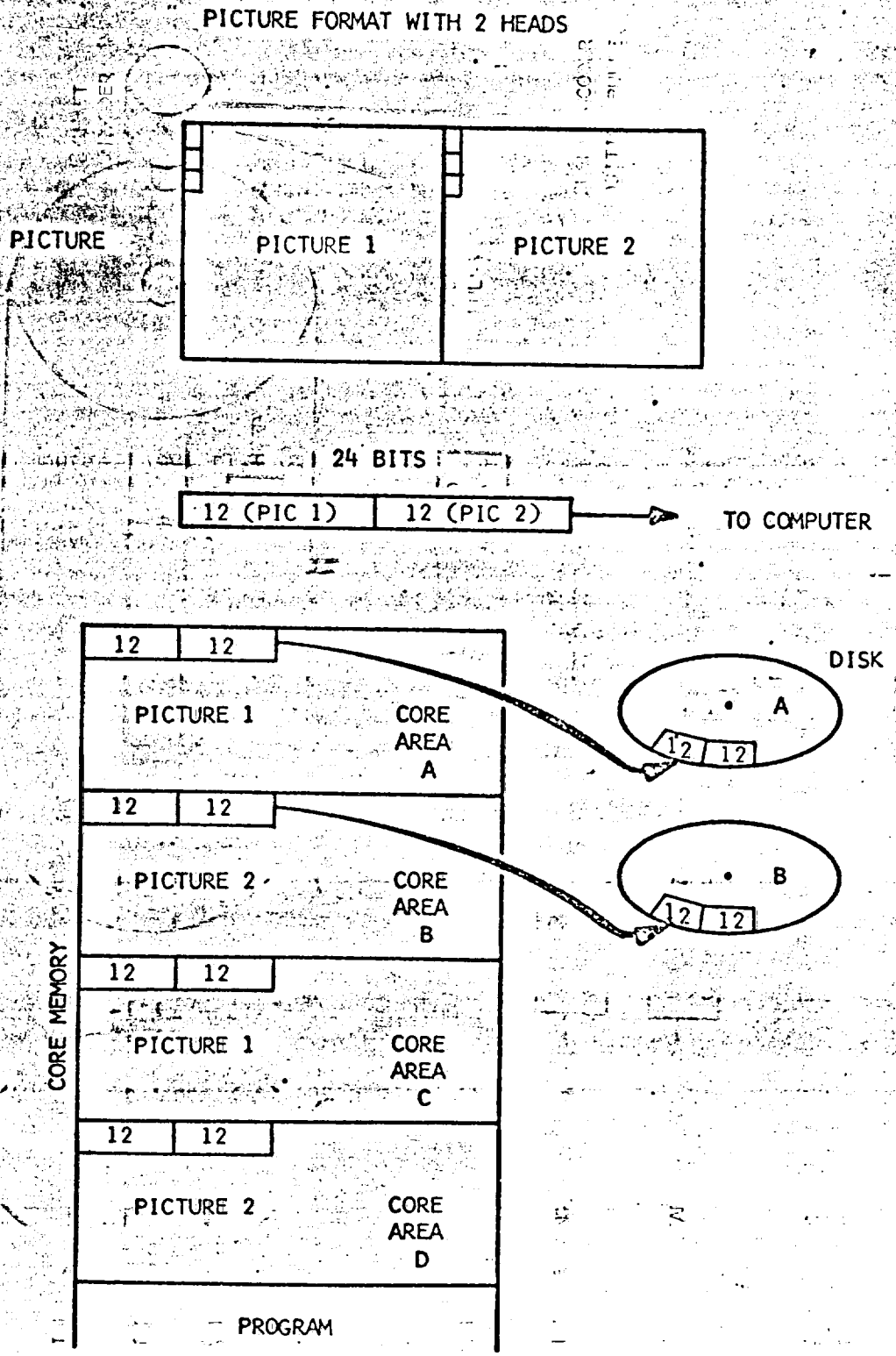


Fig. 9. Illustration of System for Interleaving the Two Halves of the Picture onto the Disc.

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(a) FIDACSYS (FIDAC system). This system inputs the images into the computer from the scanning devices, and accomplishes overall picture manipulation and enhancement. The list of options and subroutines is too lengthy to give here, but we will illustrate a typical set of options available for reading from FIDAC or VIDIAC into the computer.

One part of the system is a general utility program for handling FIDAC pictures conveniently. It may be used to read FIDAC, VIDIAC, or DRIDAC, store pictures on tape and read pictures from tape, and allows several options such as specification of bits per spot from 1 to 6, and limited printing windows designated by using the joy stick. Up to fifty character sets are stored on disk "FIDACD" and any one may be called upon to print a picture. These character sets may be altered and the complete set may be listed for examination at any time. The program can be interactive with the computer console and MACDAC, and in this mode responds to one "command" at a time, and upon completion types the word "COMMAND?" In general, each command consists of two letters and two numbers.

A typical command is CB, change bits: this allows change of spatial density read from FIDAC and number of bits per spot. Spatial density may be any integer from 1 through 15. Bits per spot may be 1, 2, 3, 4, or 6. With this information, the computer determines whether an entire picture of this size will fit in memory. If it will not fit, the computer responds by requesting a window size to be pointed out on the MACDAC scope using the joy stick. This window is designated by indicating the width desired, and the computer responds by indicating the height allowed for that width by drawing the picture limits on the scope. This procedure may be repeated until the operator is satisfied with the area he will get. When satisfied, he pushes the END button, and the window is stored in memory. Often a picture size of exact dimensions is necessary, when reading a picture from tape, which could not be easily obtained by pointing on the scope. This may be specified as follows: when the computer has requested the joy stick coordinates for the top corners desired, push the INTERRUPT button. The computer will now ask for the exact number of spots per row. The number of lines is calculated and is not under the operator's control. The END button must be pushed to accept the results of this command, unless a non-zero number is typed after spots per row is typed.

Other commands are: CCnn, change characters; CS, change size; CW, change window; PC, print characters; WF, write on film in FIDAC (i.e., make a grey level negative on the FIDAC used as a "reverse" scanner); RFnn, read FIDAC; RTnnKK, read tape; SP, spectrum point; PPnnKK, print picture on high speed printer with a designated character set; WSnn, write on the MACDAC display unit; WTnnKK, write tape, and so forth.

For reading very large pictures (i.e., raster sizes of 2,000 x 2,000 spots) a special subprogram is required as follows. The subprogram "FIDPIC" reads a FIDAC picture into memory from the FIDAC, but retains only a specified window in memory. To do this, the program constantly monitors the address into which the picture is being read; when an undesired portion is being read, this address is altered to reference a "discard" area and that portion of picture is thus destroyed. Using this technique, the picture can be read, portion after portion, on successive scans, each scanned region stored on disc and thereby assembled as a complete picture which would have been too large to be stored in memory in its entirety.

Programs "FIDIND" and "REDUCE" are used in conjunction to read a 4 bit/spot picture from disc and convert to 2 bits/spot in memory with the resultant loss in grey levels, but gain in picture size capable of being stored.



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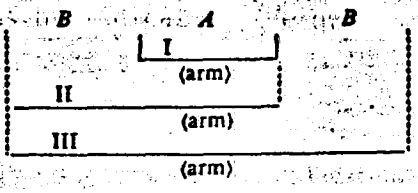
(b) SYNTAXSYS and BUGSYS Pattern Recognition Languages. These two systems present a large variety of basic pattern-recognition capabilities, including syntax-directed pattern recognition, feature-recognition methods, boundary-characterization techniques, etc. We have published extensively in the literature on these languages.

The SYNTAXSYS language uses an approach to pattern recognition based on some of R. S. Ledley's early original research into picture grammars. Consider a characterization of the boundary of an object in terms of five types of curves: a clockwise curve, type A; a relatively straight line, type B; a counterclockwise curve, type C; a notch, type D; and a wide clockwise curve, type E. Syntactical definitions of the different kinds of chromosomes can be made in such terms as these. A boundary is first characterized as a list of such curve types, and then the syntactical definitions are used to "build up" derived parts of a chromosome from combinations of these curve types. For example, the syntax for a submedium chromosome and a telocentric chromosome is shown in Table 1. The recursive definition

<arm> :: = B <arm> | <arm> B | A

means that the generic concept *arm* is defined as being a B type followed by an arm, or an arm followed by a B type or an A type. In this notation, the angular brackets < > indicate that a generic name is enclosed, the ":: =" means "is defined as being," and the "|" means "or." The definition is recursive in that it is used repeatedly. For example, consider our definitions applied to the figure ABC as follows:




where we first (I) identify the A type as an *arm*, and then build up the arm by repeated application of the definition to include (II) the B type on the left and (III) the B type on the right. Further consideration of these concepts can be found in the references. (See Fig. 10.)

The process is, of course, systematic, and is carried out by a special program in the SYNTAXSYS system called the MOBILIZER [which is directly analogous to the "translator" of automatic-programming-language translation]. The mobilizer operates on a parts list for a particular object, and by using the generic syntactical description of various kinds of chromosomes "recognizes" the object as a chromosome or not a chromosome, and if a chromosome is recognized, as a particular kind of chromosome. Actually the mobilizer works with numbers: as shown in Table 1 the syntax can as well be written in terms of level numbers, where the numbers are assigned to the left-hand entities in the order in which they appear in the syntax. The basic parts, namely A, B, C, D, and E, are always considered to have number values less than any generic or "derived" part.

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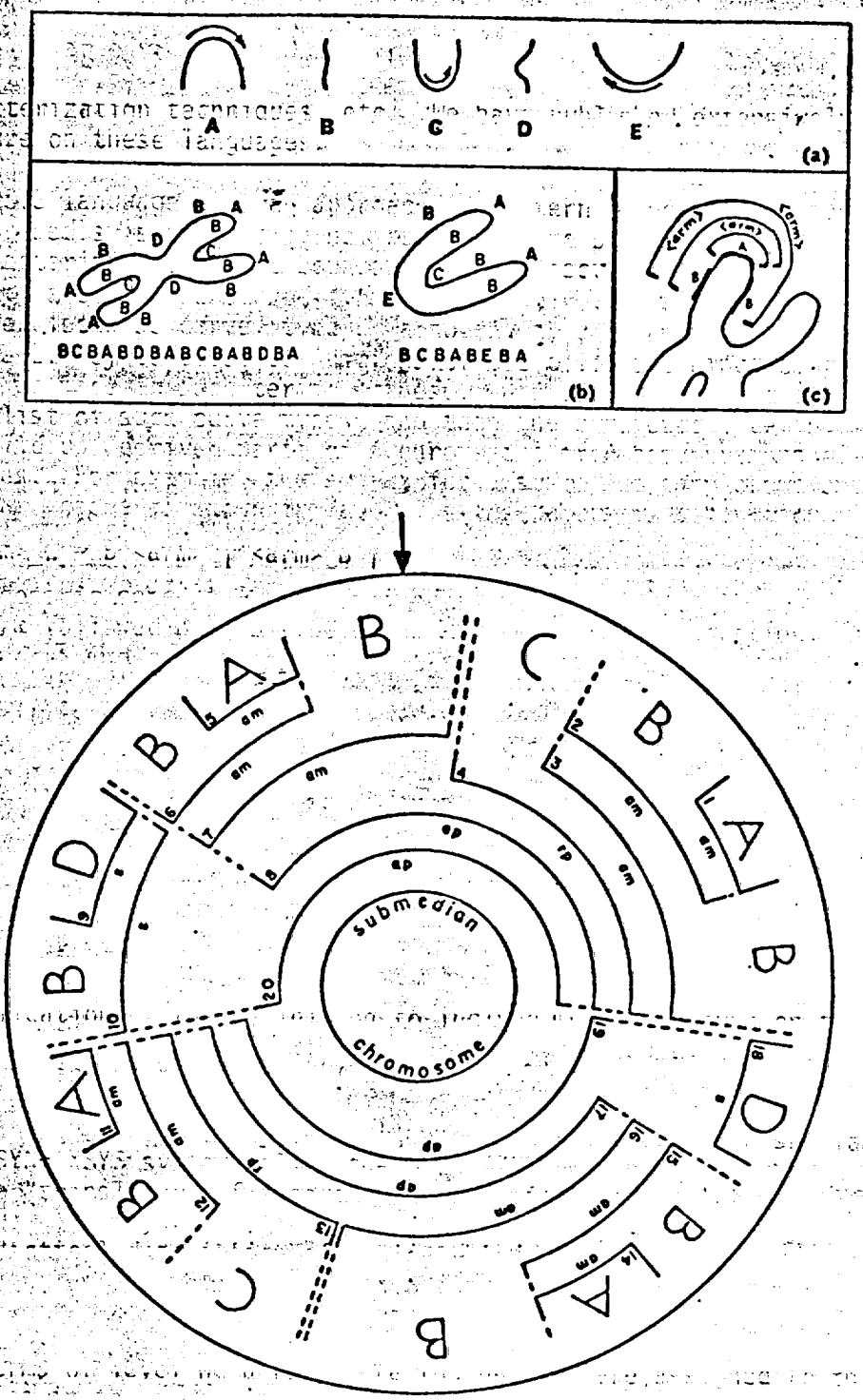


Fig. 10. (a) The basic types of curves used for the illustration in the text.

(b) Illustration of submedian and telocentric chromosomes.

(c) Short example used in text.

(d) Example of syntactical analysis or the build-up process followed in chromosome recognition for the chromosome shown in (b). Here *am* stands for <arm>, *rp* for <right part>, *ap* for <arm pair>, and *s* for <side>.

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Table 1. Sample syntax for submedian and telocentric chromosomes.

| | |
|---|---|
| ⟨arm⟩ ::= *B* ⟨arm⟩ \| ⟨arm⟩ *B*\|*A* | 70 ::= *B*,70\|70,*B*\|*A* |
| ⟨side⟩ ::= *B* ⟨side⟩ \| ⟨side⟩ *B*\|*B*\|*D* | 71 ::= *B*,71\|71,*B*\|*B*\|*D* |
| ⟨bottom⟩ ::= *B* ⟨bottom⟩ \| ⟨bottom⟩ *B*\|*E* | 72 ::= *B*,72\|72,*B*\|*E* |
| ⟨right part⟩ ::= *C* ⟨arm⟩ | 73 ::= *C*,70 |
| ⟨left part⟩ ::= ⟨arm⟩ *C* | 74 ::= 70,*C* |
| ⟨arm pair⟩ ::= ⟨side⟩ ⟨arm pair⟩ \| ⟨arm pair⟩ ⟨side⟩ | 75 ::= 71,75\|75,71 |
| \| ⟨arm⟩ ⟨right part⟩ \| ⟨left part⟩ ⟨arm⟩ | \|70,73\|74,70 |
| ⟨submedian chromosome⟩ ::= ⟨arm pair⟩ ⟨arm pair⟩ | 76 ::= 75,75 |
| ⟨telocentric chromosome⟩ ::= ⟨bottom⟩ ⟨arm pair⟩ | 77 ::= 72,75 |

V

The BUGSYS language is a picture-processing and -measuring programming language for the analysis of the picture in the computer's memory. The main concept of the system is the use of a collection of programmable pointers, which are visualized as a family of "bugs." A bug can be "initiated," or "placed," and once initiated a bug can be "moved." In addition, a bug can "change" the grey-level value of the spot on which it is located, and it can lay down a so-called "stick" across a thick line in the picture as an aid to locating the middle of the line. Also, two bugs together can "probe" along the direction of the line between them or along a direction perpendicular to the line between them. These probes can sense an extension of an object or the width of an object, and so forth. "Globs" of objects can be assessed by laying down squares and determining the percentage of the area of the square intersected with the object. The system is composed of many such statements as will be illustrated below. (See Fig. 11.)

For each bug initiated by means of the macro PLACE, there is associated a list which gives the x and y coordinates of the current position of that bug, the actual core location, the spot position within this location, and the grey-level value of the current position of the bug. For example, if the bug named "ZIPPY" is in the position x = 16, y = 8, then the list for ZIPPY might contain the following.

| Address | Contents | | Comment |
|---|---|---|---|
| ZIPPY(1) | 16 | *x* | |
| ZIPPY(2) | 8 | *y* | |
| ZIPPY(3) | 6351 | 6000 | (first location of picture) |
| | | +350 | [50 words per line × (*y* − 1)] |
| | | +1 | (*x* divided by 10 spots per word and truncated to an integer) |
| ZIPPY(4) | 6 | | remainder of *x* divided by 10 |
| ZIPPY(5) | 4 | | gray-level value or contents of spot |

As a bug is moved about the picture by a program, the list of the bug is kept current. In fact, in essence, this list *is* the bug.

We will now proceed to describe some of the statements of the BUGSYS language. The PLACE statement initiates, or sets up, a bug by assigning a name and initial coordinates to it. Thus we have

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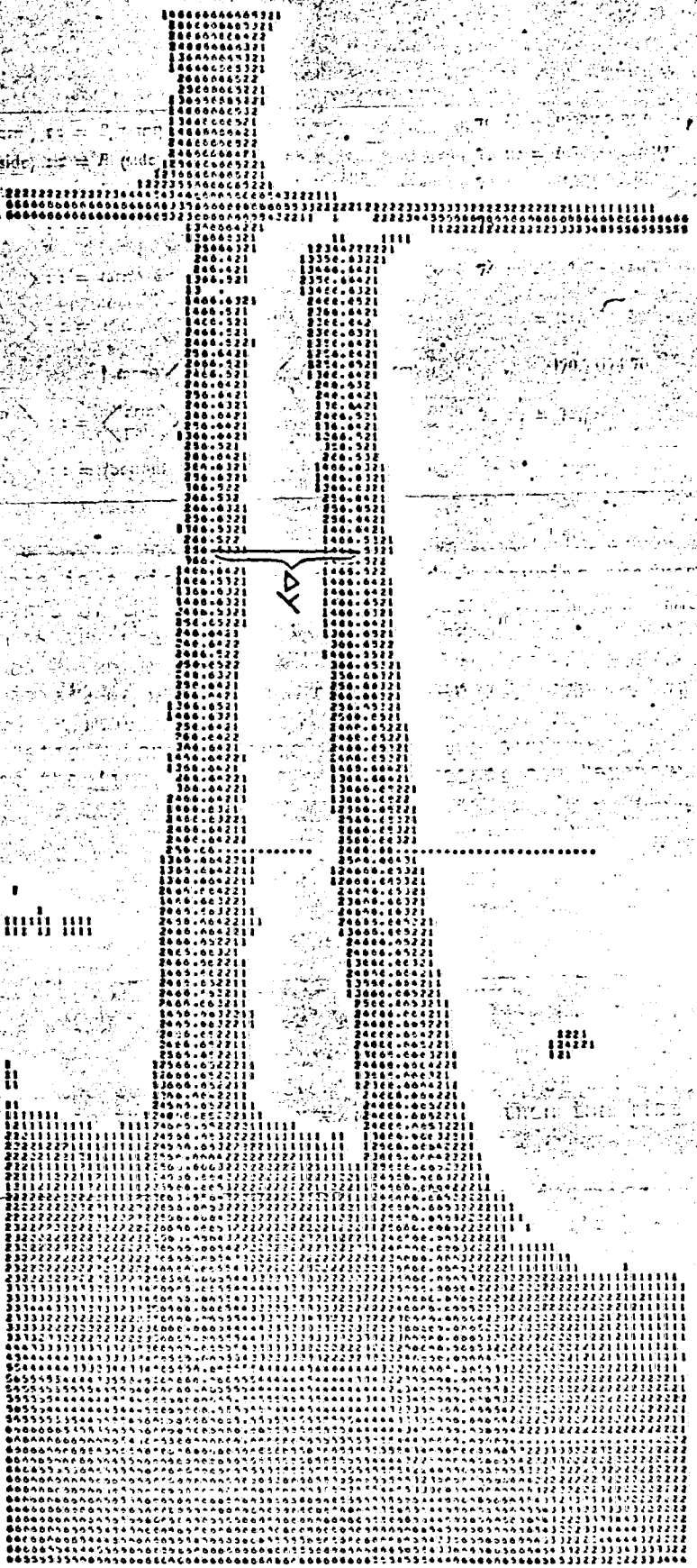


Fig. 11. Illustration of the Application of BUGSYS to Determining Successive y Coordinates of a Curve (taken from a Schlieren photograph). The dots illustrate the path of the bugs taken in making the successive Δy measurements.



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<PLACE statement> :: = PLACE <bug name>, <x coordinate>, <y coordinate>

where the bug name is a FORTRAN label and the x and y coordinates are either unsigned integers or integer variables.

The BUGS statement allocates five storage locations to each bug named; its form is

<BUGS statement> :: = BUGS (<bug-name list>)

where the bug-name list is a string of bug names separated by commas.

A bug can be moved a specified distance (i.e., number of spots in the picture) in either the x or y direction by the following statement.

<MOVE statement> :: = MOVE <bug name>, <direction>, <distance>

where the direction is given by the literals LEFT, RIGHT, UP, or DOWN, and the distance is an unsigned integer or integer variable. The statement "MOVE ZIPPY, RIGHT, 15" moves the bug named ZIPPY to a new location having the same y coordinate but a new x coordinate 15 spots to the right of the present x coordinate. Each time a bug is moved, of course, the list corresponding to the bug name is adjusted for the new values.

Many of the statements involve multiple-way branches, for provision must be made to be sure that the bug will not be moved out of the picture. For instance, in the MOVE statement if the bug would be moved out of the picture, then it is not moved at all, and the next sequential instruction is taken as a next executed instruction. Otherwise (i.e., if the bug will still be in the picture after the move) the bug is moved and the next sequential instruction is skipped. The BUGSYS language also includes provisions for bounding an object. This is the same subprogram as the bounding routine which appears in the SYNTAXSYS system, except that in BUGSYS it can be utilized in a macro called BOUND.

(c) MACDACSYS (MACDAC system). This programming system implements the interactive capabilities of the MACDAC unit, including interrupt features for accepting information from, and displaying pictorial and alphanumeric information on, the MACDAC unit. Typical examples of operator interactive capabilities provided by the MACDACSYS system are as follows. Utilizing the joy stick in either regular or zoom mode, the operator can connect two points, erase an object, slice an object into two parts, and draw a "fence" or line around a collection of objects that are to be erased from the picture in the computer's memory. Also the "end of job," the "end of codes," and an "erase last code put in" commands are available. In many interactive-operator procedures, the objects displayed on the MACDAC display are numbered. The operator can point to one of the numbers to direct the computer to process in some way the associated object. To do this the joy stick is positioned with the cursor near the number and the "read spot" button is pressed. If the computer's bell rings (the IBM 360/44 has a programmable bell) then this tells the operator that the cursor is not close enough to the number. This method of having the computer signal the operator by means of the bell, or a series of coded bell rings, is used extensively in our interactive system.

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The MACDACSYS system is designed for capability of running in a time sharing or overlapping mode. In this mode, the picture being worked on has been placed on the MACDAC display directly from the scanners, and not from the computer. Thus the computer can be carrying out an entirely different computation with no picture at all, or a different picture, in its memory. The editing or interactive procedures carried out by the operator are stored in the computer's memory, the MACDACSYS interrupting the computer's computations from time to time as the operator is working. Then, at a later time when the operator actually scans the picture into the computer's memory, the directions given previously by the operator for editing the picture are now carried out.

(d) SPIDACSYS (SPIDAC system). This system automatically directs the motion of the SPIDAC microscope stage, detects good chromosome spreads, and records the coordinates (to the nearest 1.25μ) of the center of each such good chromosome spread. It also relocates the microscope stage at such coordinates, and directs the vidicon scan into the computer.

The SPIDACSYS software package consists of two parts. The first part is pertinent to the covering, detection, and locating centers of the objects of interest (e.g., chromosome spreads) during the search mode. The second part controls the stepping motors and moves the microscope stage such that these objects are centered automatically for detailed analysis during the analysis mode.

The complete operational procedure is as follows: The operator or the technician first puts the glass slide onto the microscope stage, selects the origin (usually left-upper corner of the cover slip), and turns to the low power object lens. At the same time, he loads the software package into the computer. When everything is ready, he pushes the start button and waits. Meanwhile, the hardware searches the successive fields of a strip, and then goes to the next strip, etc. until the forty strips have been searched field by field. In between searches the software program finds covers and determines centers of the objects of interest (e.g., chromosome spreads) in each field, and this information is saved for the analysis mode. An optional visual display is also included in the software program. This visual display puts all the coverings and domains of chromosome spreads on the MACDAC scope (or TV screen) for each field. (See Fig. 12.)

Next the operator or computer switches the objective lens from low power to high power. He then pushes the start button for the analysis mode. The computer program sends a signal to the hardware to move the microscope stage so that each of the objects found is successively centered under the objective lens. The hardware sends the detailed scan of each such object one by one onto a TV screen. The technician judges from the TV screen the acceptability of the object for analysis. If it is a bad one, he may reject it and the next object will be shown. If it is a good one, he may push the analysis button. This enables the fine scan of the object to be made and the object is analyzed by the computer. Then the next available object will be displayed. This procedure continues until all the objects have been processed. The software program then moves the microscope stage back to its original position and terminates the procedure.

(e) DOCS (Display Of Chromosome Statistics). The DOCS programming system enables on-line computer-console interaction with the disk memories of the computer for the evaluation and display of large masses of data in

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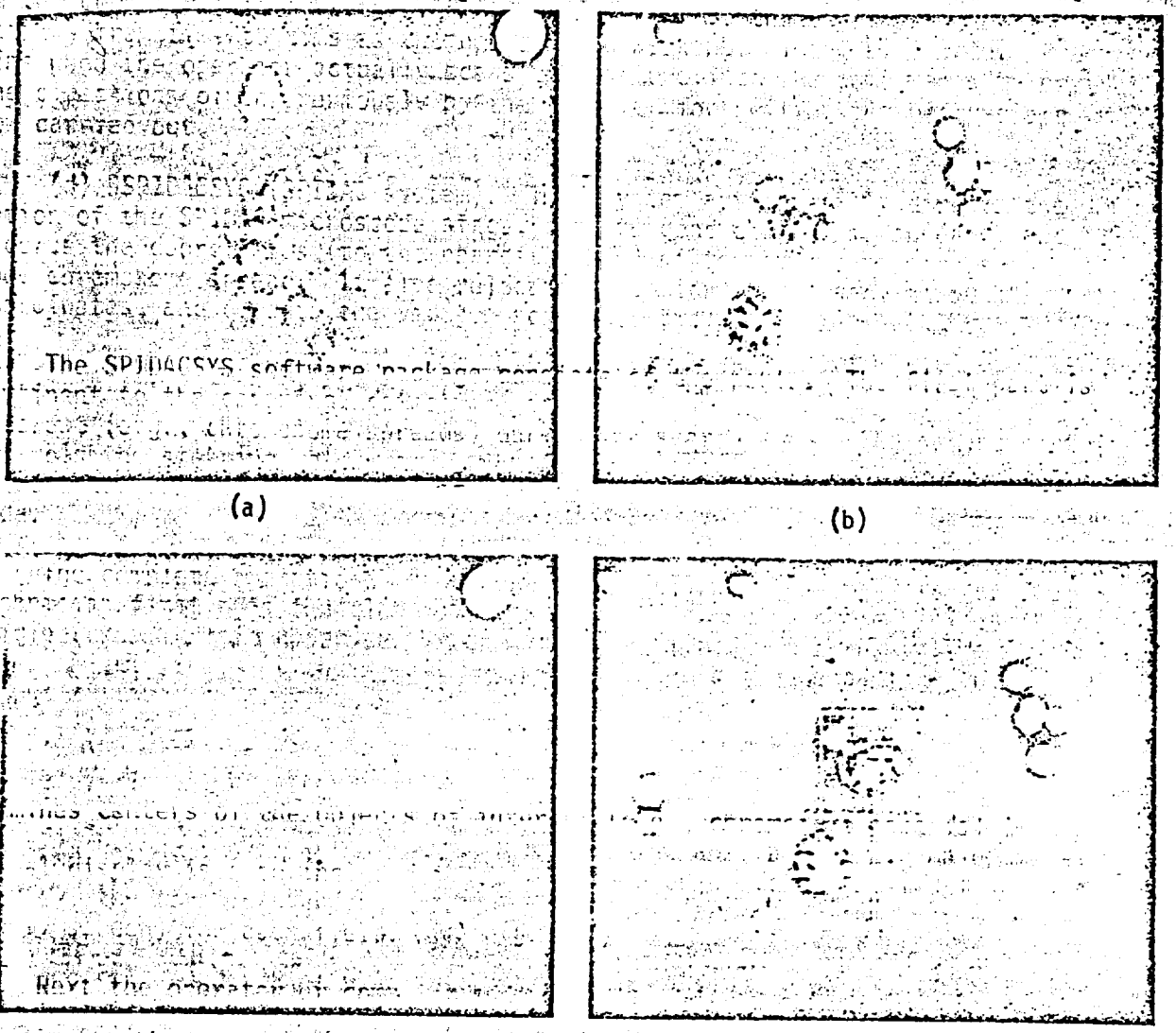

Fig. 12. Application of SPIDAC to Detecting Chromosome Spreads.

(a) Chromosome spread as seen on MACDAC display tube.

(b) Horizontal lines illustrate the recognition of the portion of a chromosome spread.

(c) Two chromosome spreads in a field.

(d) Result of computer locating each of the chromosome spreads.

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a file. For instance, analyses of variance, t-tests, and other statistical tests can be executed on data selected from the file. The DOCS system guides the user in a step-by-step dialogue, so that all of the detailed specifications required for the desired statistical analysis or data display can be obtained.

(f) REMOTE. This programming system enables the remote user to be serviced by the computer.

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1. R. S. Ledley, Programming and Utilizing Digital Computers, New York: McGraw-Hill, ch. 8, 1962.

2. R. S. Ledley, M. Legator, and J. B. Wilson, "Automatic Determination of Mitotic Index," in Pictorial Pattern Recognition, G. C. Cheng et. al., Eds., Washington, D. C.: Thompson Book Co., 1968.

3. R. S. Ledley, "High-Speed Automatic Analysis of Biomedical Pictures," Science, vol. 146, no. 3641, pp. 216-223, October 9, 1964.

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6. T. Golab, R. S. Ledley, and L. S. Rotolo, "FIDAC - Film Input to Digital Automatic Computer," Pattern Recognition, vol. 3, pp. 123-156, 1971.


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ADDENDA

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Addendum 1

COMPREHENSIVE REPORT ON THE ACHIEVEMENTS OF THE "GROUP"

Historical:

The origin of the group goes back to the summer of 1972 when Paul Hamosh transferred from the Veterans Administration to Georgetown and got together with Dr. Vidic to establish a group dedicated to "Structure and Function of the Lung." In the Fall of 1972, Dr. Bellanti contacted Dr. Hamosh and informed him about his intentions to apply for a Program Project in Lung Immunology and invited him to participate. Dr. Hamosh also recommended Dr. Vidic, as a participant, and a basis for close co-operation was established.

However, not all activities were immediately integrated. In January 1973, Dr. Hamosh became the recipient of an award from the Center for Tobacco Research for the study of "Smoking and the Small Airways." This study was a continuation of research performed by Dr. Hamosh and Dr. Da Silva with the support of the Veterans Administration ( Dr. Da Silva was a fellow in pulmonary medicine at the VA hospital at that time).

It became soon clear, that without a biochemist, the research on structure and function has no depth. Application was made for a second Center grant: "Smoking and Lung Metabolism" in which provisions were made for hiring a biochemist. The proposal was funded 23 January 1974 and by July 1974 we succeeded in recruiting Dr. Margit Hamosh who spent the previous nine years at the National Institutes of Health. We were also lucky in acquiring the services of Dr. Schechter, a biochemist with considerable experience who, unfortunately for us, will return to Israel in the near future.

In the meantime, even before funding by CTR, the group started working together with the aid of the Washington Heart Association. Most of this work was centered around the incorporation of radio-labeled palmitate into isolated perfused lungs as a function of ventilation. During the early stages of research in 1974, it became clear that biochemistry of tissue slices or homogenates or even isolated perfused lungs might yield answers to complex problems requiring pure populations of cells. Indeed, the arrival of Dr. Jean Wrathall in January 1975 was greeted with great enthusiasm. We gained a tissue culture expert of excellent background (Cornell University) and experience. At about the same time, we discovered that Dr. Goeringer, who just returned from a sabbatical leave at Oxford, has extensive experience in cell separation techniques and organ cultures. The addition of these two people to the group rounded and expanded the scope of our activities.

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Another significant development was the realization that with the expanded workload, Dr. P. Hamosh could not perform his research in physiology without significant input by an additional skilled investigator. Fortunately for the group, Dr. Da Silva, who spent two years in Portugal, agreed to join us, which he did in the Fall of 1974. The most recent addition to the group, Dr. Rabinowitz came to us when we started work on human lung tissues. It became clear, that without the cooperation of a pathologist, such a project will not get off the ground. Thanks to him , we have now a steady flow of specimens.. He is also interested in directing our effort in morphometry of the human lung.

The group is now a well rounded one and further additions will be on the "junior" level. We envision a number of post-doctoral fellows working under the supervision of the investigators. The program project will now provide the backbone of this organization.

The Pulmonary Program Project, the one directed by Dr. Bellanti, is centered around the role of the macrophage in the defense of the lung. It provides, in addition to cooperation in sharing resources (technical help and specimens), a contact point through project #4 (Maturation Studies of Cellular and Immunological Processes in Pulmonary Defense Systesm). Drs. Vidic and M. Hamosh are active co-investigators in that project..

The group has considerable outside support. Most significant are the computer support of the National Biomedical Research Foundations and within it a Princeton educated computer expert Dr. Robert Shile. Dr. P. Hamosh is still supported by the Veterans Administration. Excellent collaborative arrangements exist between different laboratories at the National Institutes of Health and the group (in NHLI, NIAMDD and NICHD). The following scheme indicates the historical development of the group and some of its present intra and extra-mural activities.

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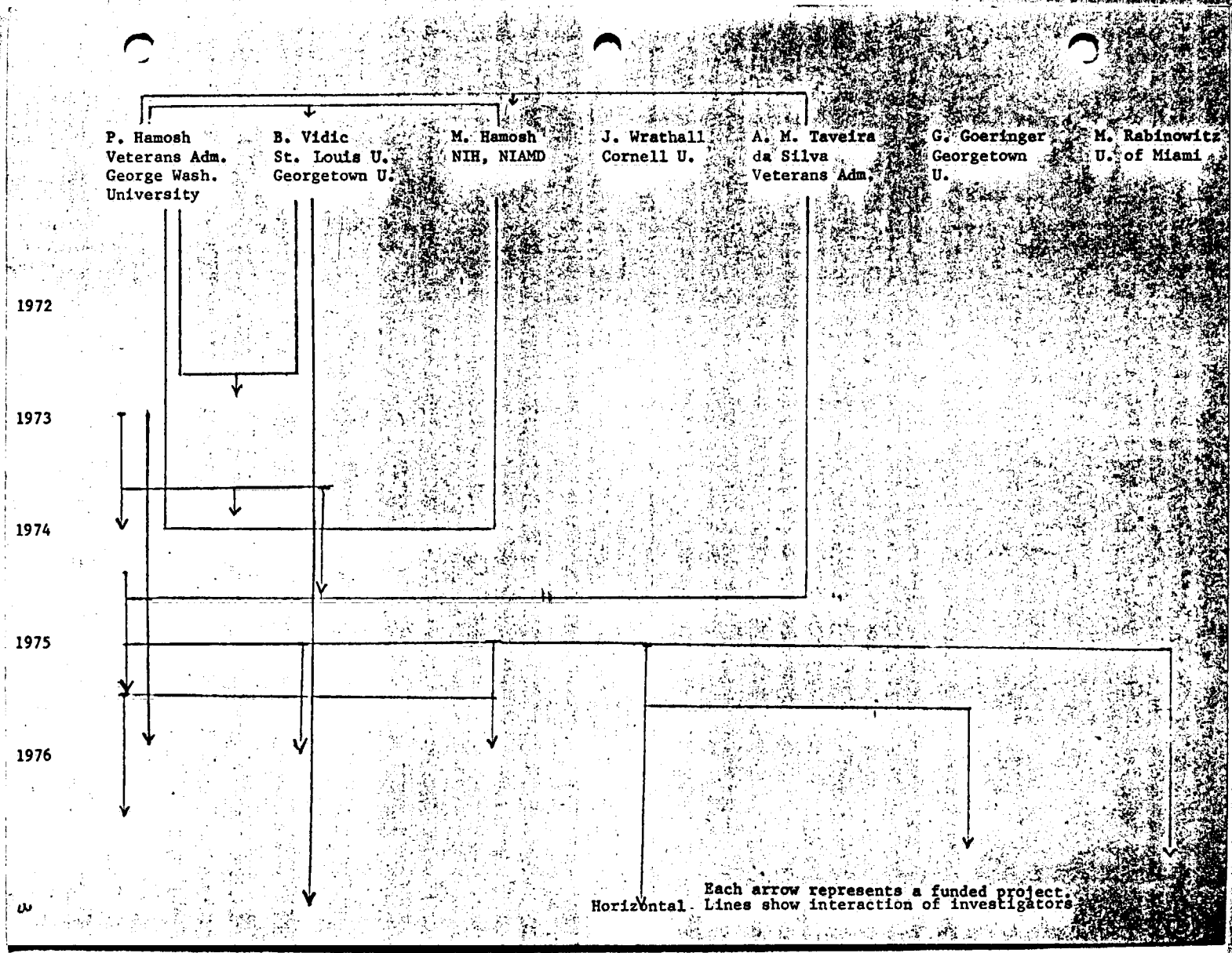


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Achievements of the Investigators from 1972.

Since the inception of the groups' activities, P. Hamosh's main thrust was the establishment of the framework for a major interdisciplinary effort in lung research. The goals were to 1) establish a laboratory in respiratory physiology 2) find a good structural investigator 3) attract a good biochemist who will establish a laboratory in lung biochemistry and 4) attract funds for research.

The first goal was achieved by maintaining the support of the Veterans Administration and keeping the equipment bought in the years 1970-1972. This led to completion of projects and three publications:

1. The Effect of Smoking a Single Cigarette on the Samll Airways. J. Appl. Physiol. 34:361, 1973.
2. Supine Hypoxemia and Erythrocytosis due to Air-Way Closure at Low Lung Volumes. Am. J. Med. 55: 80, 1973.
3. Air Bolus Method Compared to Simple Breath Method for Determination of Closing Volume. Am. Rev. Resp. Dis. 110: 518, 1974.

All this work was performed partially in the Veterans Administration and with the participation of Dr. Taveira Da Silva, who was trained by P. Hamosh. We also continued work on shearing stress which led to the presentation of the work in Cincinnati at the American Thoracic Society's annual meeting (The Effect of Shearing Stress on the Bronchial Mucosa. Am.. Rev. Resp. Dis. 109:694, 1974. and the manuscript (submitted in its present form to the CTR with a preliminary proposal). The human studies (Smoking and the "Small Airways") received the support of the CTR beginning January 1973 and has been pursued ever since. The arrival of Dr. Taveira Da Silva in late 1974 gave a tremendous impetus to this work. We are in the process of summarizing the results of the first two years of this research. We have asked basically two questions: Can one show after acute smoking ("provocation test") different responses in subjects, who might then be "reactors" or "non-reactors" to acute inhalation of cigarette smoke? The second question was more specific: what test to use for this discrimination? In the course of this research we produced data which are now being assembled into three manuscripts:

A. "The time course of response to smoking a single cigarette." We found that 1) response to smoke inhalation is maximal after just three puffs and remains plateaued irrespective of the amount of further smoking. 2) There is a quicker response of the large airways (airway resistance) and a

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slower in the "small airways" (maximum expiratory flow at low lung volumes) which is also more persistant in the latter 3) the response is individual and a "responder" will respond always with the same quantitative change.
B. "The most sensitive test for differentiating smokers from non-smokers: the $MEF_{50}$."

We have compared in a large series of young subjects all accepted pulmonary function tests and found only the $MEF_{50}$ useful in differentiating with acceptable probability ($P < 0.02$). We used a great number of manipulations of the curve ($MEF_{50}$, $MEF_{25}$, MMFR, $MTF_{50-75}$ and $MTF_{75-85}$ etc.) and compared also water spirometer to dry spirometer to body plethysemsgraph. This latter part of work will probably be published separately.
C. "The effect of smoking a cigarette on the Helium Expiratory Curve". This work is essentially a study on the location of the equal pressure point (EPP) and deals with the mechanism of action. The results are negative and cast some doubt on the value of the helium curve.
At the time of writing we are engaged in two experimental protocols: 1) Racial differences in response. Recent work shows that in blacks it is difficult to separate smokers from non-smokers on the basis of pulmonary function tests. We found that their acute response to cigarette smoke does not differ from the whites. This is a very important finding and we are in the process of collecting enough data to achieve the level of statistical significance necessary for publication.
2) We have done work on using different Kentucky brand Research cigarettes and comparing the response. The response is definitely dose dependent (1R1 is higher that 1A1). 3) we are about to start to study the effect of smoking a cigarette on regional lung function using $^{133}Xe$.

We hope to go on with this research within the framework of the program project after 1975 (project #1D).

In January 1973 we received a grant from the Washington Heart Association for the investigation of the effect of ventilation of lung surfactant metabolism. This was followed by a second award with Dr. Vidic as Principal Investigator in July 1973 and a third in January 1975. These grants were the initial resources on which cooperation between P. Hamosh, B. Vidic and M. Hamosh was based. M. Hamosh started working with us on weekends, when still at NIH. The project was essentially the study of the incorporation of $^{3}H$ palmitate into isolated pefused lungs ventilated at different rates and tidal volumes. This work was presented at the meeting of the eastern section

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of the American Thoracic Society in Washington, D.C., 1974. (The effect of Ventilation on $^{3}$H-palmitate uptake by isolated perfused rat lungs. Proc. A. T. S. 1:3, 1974), and some other aspects of it at the annual meeting of Anatomists in San Diego in 1974 (same title, J. Cell Biol. 63:359a, 1974).

P. Hamosh provided the physiology, while Dr. Vidic the autoradiography and Dr. M. Hamosh the biochemistry. This work will also provide the substance of Mr. Michael Blunda's Ph.D. thesis, who was trained by Dr. P. Hamosh.

Using the resources from above grant and in collaboration with Drs. Yeager (from the other Program Project) and Dr. Schechter, the hormonal control of lipoprotein lipase in lung was investigated in rats. This manuscript is now in preparation. Other work done on lipoprotein lipases was all in the framework of this preliminary studies which were fleshed out with the beginning of additional support by the CTR "Smoking and Lung Metabolism: awarded to Drs. Vidic and Hamosh in January 1974. Therefore, during 1973 the second goal was achieved by the inception of cooperation between Drs. Vidic and P. Hamosh. The third goal was achieved in June 1974 when Dr. M. Hamosh joined full time the Department of Anatomy at Georgetown University and became a full partner in the group. Most of the work mentioned above will be described in detail in her narrative, since she is the principal author.

In 1975 two more grants from the Washington Heart Association are supporting the work on Lipoprotein Lipase. One was awarded to Dr. M. Hamosh as principal investigator to pursue the finding on the effect of estrogen in the rat and apply them to humans. The other was awarded to Dr. Paul Hamosh to test his hypothesis of the possible role of chylomicrons in atherosclerosis. This latter work combines the research on vascular shearing stress with the research on lipoprotein lipase. It is worthwhile to mention that the Hamoshes collaborated successfully already in the early sixties (J. Clin. Invest. 42: 1648, 1963).

Collaborative work between P. Hamosh and Vidic has led to a number of projects in progress. Most of these are centered around the morphology of surfactant (see manuscript submitted with progress report 936R2).

P. Hamosh has also done cardiovascular work during this time, mostly with the assistance of A. da Silva. Thanks to a grant from the Washington Heart Association, the effect of volume loading on lung compliance was studied. This experimental design was also used to complete a study on the mechanism of hepatojugular reflux which was submitted for publication. With Dr.Kulczycki from Children's Hospital, work was done on children with cystic

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fibrosis and the results were published in the Cystic Fibrosis abstracts (1974). In cooperation with Dr. Ungerleider for the National Biomedical Research Foundation a computer program was worked out for automated assessment of emphysema. With Dr. Da Silva, a drug study was completed on the effect of prostaglandin $F_{2\beta}$ on the airways (submitted to "Prostalgandins" for publication). A computerized ventilation performance scan on cancer patients is in progress funded by a contract with the Veterans Administration.

All these activities led to the achievement of the fourth goal; attracting funds for a relatively large scale operation. The new goal is to expand and unify these activities under one single effort toward which goal the program project was directed.

M. Hamosh

Early work on fatty acid release from the liver (1961) and on the hormonal regulation of fatty acid release from adipose tissue, (1960) led to the first joint study of M. and P. Hamosh on lipid metabolism in human adipose tissue (1963). This was one of the first studies in the human and showed that there are marked differences in fatty acid metabolism between the "internal" (omental, perirenal ) and subcutaneous adipose tissues. The metabolism of adipose tissue was further pursued by M. Hamosh with special emphasis on changes leading to the development of obesity (1964). In 1965 M. Hamosh started a two year sabbatical at the NIH; she decided to broaden her experience and to spend this time in studies related to protein and nucleic acid metabolism. The result of these studies was the discovery of enhanced protein synthesis in a cell-free system prepared from skeletal muscle undergoing rapidly compensatory hypertrophy (Publications: 1967, 1968). The years 1968-1974 were spent at the NIH. During this period the major interest was in the regulation of lipid transport and the digestion of dietary fat. The uptake of circulating lipid (which is present in the blood chiefly as triglyceride in chylomicrons and very low density lipoprotein) is catalyzed and regulated by the enzyme lipoprotein lipase. Research was therefore centered around this enzyme, with the specific objective to elucidate the relationship between the lipemia of pregnancy and the activity of this enzyme. The major findings were that the hormone prolactin induced changes in the level of lipoprotein lipase activity in adipose tissue (inhibition) and mammary gland (stimulation). These changes in enzyme activity channel dietary triglyceride from storage in adipose tissue to milk formation in the mammary gland (see references). The presence or absence of lipoprotein

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lipase in the milk of the lactating animal can be taken as a measurement of holocrine or apocrine secretion (see reference 1971).

The mechanism of LPL activity was studied in the isolated, perfused adipose tissue and mammary gland, by biochemical and especially developed electronmicroscopic cytochemical methods Studies on the digestion of dietary triglyceride were initiated when it became apparent that the low pancreatic lipase activity of the newborn is insufficient to digest the large amounts of fat present in milk. These studies led to the discovery of a new digestive enzyme, that is secreted from lingual serous glands (Von Ebner) and is active in the stomach in the digestion of dietary TG to partial glycerides and FFA: A similar digestive enzyme has subsequently been described in the human. EM and biochemical studies conducted jointly with Dr. A. R. Hand are now in progress on the development of secretory activity in rat lingual serous glands These studies show that secretory activity (measured by the present of lingual lipase) can be detected 2 days before birth in the rat. At this age the lingual serous glands are not yet developed, whereas the mucous glands are well developed. In the suckling and adult rat, lingual lipase is secreted by serous glands, it seems therefore, that during early development the enzyme is secreted by demilune cells (serous cells present within mucous secreting glands).

Accomplishments since 1973 with P. Hamosh.

In 1973 M. Hamosh was invited by P. Hamosh to help set up the methodology for biochemical studies of the lung. These studies can be divided into 3 parts:

1. The study of lung metabolism in the isolated perfused lung.
2. Lipoprotein lipase regulation of activity and role in lung metabolism.
3. The effect of smoking on lung metabolism - to be studied in the following tissue preparations: isolated perfused rat lung; lung slices and isolated cultured type II alveolar cells.

The questions which we want to answer using the isolated perfused lung are:

a. what is the relationship between ventilation and the synthesis ( and possible use) of surfactant.
b. Are there changes in the synthesis and secretion of surfactant as a function of vascular perfusion.
c. Does exposure to air pollutants (i.e.: cigarette smoke) change the ability of the lung to synthesize surfactants (at normal ventilation or during hyperventilation).

The first part (a) of the study has been completed and theresults were presented by Dr. M. Hamosh at the American Thoracic Society Meeting in October 1974. Briefly, we have found that ventilation at high tidal

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volume (40% of TLC) doubles the uptake of infused $^{3}$H-palmitic acid and its incorporation into phosphatydilcholine. Thus palmitate incorporation into phospholipids (surfactant) is a function of respiration. Autoradiography of the perfused lung shows a faster flux of the label from endoplasmic reticulum through lamellar bodies and onto the alveolar surface at high tidal volume (TV) ventilation. Only in the high tidal volume ventilated lung does one see silver grains associated with myeline figures (presumably "used" surfactant) in the alveolar space (ref. 1974). We are now gathering data on part b. of this topic, namely we study the effect of changes in the vascular perfusion on the incorporation of $^{3}$H-palmitate into lung phospholipids. We plan to start the last part of this project (c) very soon, and will start smoking experiments as soon as we obtain full data on our metabolic studies in smoking rat that are now in progress.

Lipoprotein Lipase

Although a great number of studies have focused on the role of free fatty acids, especially palmitic acid in lung metabolism, the contribution of triglyceride-fatty acids (the major reservoir of fatty acids) has not yet been investigated. As indicated above, uptake of blood triglyceride-fatty acids involves hydrolysis of the triglyceride to free fatty acids and glycerol; this hydrolysis is catalyzed and regulated by lipoprotein ipase.

We have shown that lung has a high lipoprotein lipase activity; we have also shown that infusion of heparin into isolated perfused rat lungs causes the immediate release of the enzyme, indicating its presence in or near the capillary wall. These findings indicate that the lung is able to utilize blood triglyceride. In addition we have shown that contrary to the enzyme in other tissues, enzyme activity in the lung remained high during fasting 8-72 hours. This indicates that, during food deprivation the lung and heart are the only tissues able to utilize circulating triglyceride-fatty acids. This work was presented at the annual meeting of the American Physiological Society, Atlantic City, 1974, followed by publication in Biochim. Biophys. Acta 380: 132, 1975. We have pursued the study of lipoprotein lipase with special emphasis on the hormonal regulation of enzyme activity in the lung.

Our studies have shown that the lung maintains a high level of lipoprotein lipase activity under physiological and pathological conditions known to decrease enzyme activity in other tissues. The manuscript of this study is now in preparation. In the course of this study we made the interesting observation that estrogen administration to male or ovariectomized female rats markedly decreased lipoprotein lipase activity in adipose tissue. We think,

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therefore, that the hyperlipemia which accompanies high plasma estrogen levels (such as pregnancy or contraceptive therapy) could be due to the marked inhibition of adipose tissue lipoprotein lipase activity. This work was presented at the meeting of the American Federation for Clinical Research, New Orleans, La., January, 1975, and subsequently published in J. Clin. Invest. 55: 1132, 1975. We plan to continue these studies on the relationship between high estrogen levels, hyperlipemia and adipose tissue lipoprotein lipase activity in the human (on a Washington Heart Association Grant awarded to M. Hamosh).

3. The Effect of Smoking on Lung Metabolism

a. As described above, our studies on the effect of smoking on the incorporation of palmitate into the isolated perfuse, ventilated and especially hyperventilated lung will be initiated as soon as our studies on the normal lung are completed.

b. Lung slices- In preparation for our studies on the effect of tobacco smoke on lung metabolism we have first investigated lung metabolism in normal rats. Since there is in general a lack of standardization of the methodology employed in the study of lung metabolism we have adapted the following methods to the study of lung metabolism in tissue slices:

1. Glucose Metabolism measured as the amount of $U^{14}C$ Glucose metabolized to $^{14}CO_2$ (aerobic metabolism), lactate (glycolysis) and incorporated into tissue lipid.

2. Protein Synthesis measured by the incorporation of leucine into lung protein.

3. Glucoprotein Synthesis measured by the incorporation of glucosamine.

4. Lipid and Phospholipid Synthesis measured by the uptake and esterification of labeled palmitate.

We have chosen the tissue slice method since it is the least time consuming and according to recent studies has an identical glucose metabolism as that of the perfused lung.

We have adapted the following analytical methods to the study of lung composition:

RNA, DNA, protein, lipid analysis (separation, identification and quantitation of the individual components of the major lipid classes-phospholipids and neutral glycerides).

c. Isolated type II alveolar cells by a method based on a modification of the methods of Rounds and Mason. The type two alveolar cells maintain intact, concentric lamellar bodies after isolation by gradient centrifugation. We have succeeded in culturing these cells, but at present we are worried about the loss of the typical structure of the lamellar bodies in these cultured cells. The isolated cultured cells show a corticosteroid stimulated incorporation of $^{3}H$ palmitic acid into lecithin.

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Jean R. Wrathall

From 1970 to 1974 research activites centered on the control of the expression of differentiated functions and malignant potential in cultured cells. The model system used consisted of a clone (B559) of cells derived from the B16 mouse melanoma. These cultured cells express the "normal" attributes of the "tissue" of origin.. They synthesize melanin through the production of the enzyme tyrosinase which is packaged in specialized cell organelles, melanosomes. The ultrastructure and manner of formation of the melanosomes appear identical to that of mouse melanoma cells, or normal melanocytes in vivo. Cells of clone B559 also retain their malignant potential as shown by tumor formation upon injection into syngeneic C57BL/6 mice. To probe the control of these cell functions we have been using the thymidine analogue 5-bromodeoxy - uridine which has been shown to preferentially and reversibly suppress cell-specific functions in a variety of cell types. In the melanoma cells both differentiated function (melanogenesis) and malignancy (tumorigenicity) are suppressed by concentrations of BrdU that do not significantly inhibit overall cell metabolism or growth. Besides demonstrating that malignant potential is reversible and may be regulated in a manner similar to the regulation of normal cell-specific functions, our studies have opened the way to investigating the significance of various factors (growth potential, immunogenicity, tumor viruses, fibrinolytic activity) in the overall "malignant potential" of tumor cells. Copies of representative publications (including a review article) from these investigations are included).

Since joining the Department of Anatomy of Georgetown University in January, I have been: a) establishing a laboratory in which cell tissue organ culture experiments may be successfully performed; b) continuing my research with the melanoma cell system; and c) expanding my research interests to include the regulation of lung cell functions. My present interest in lung cells has resulted from interactions with a number of Georgetown researchers involved in pulmonary research, especially Dr. B. Vidic and M. Hamosh of my department and Dr. P. Hamosh of the Physiology department. I have become convinced that study of the functions of lung cell types at the cellular level through cell culture techniques would yield worthwhile information especially as part of a integrated research program encompassing pulmonary physiology, biochemistry, development and ultra-micro-anatomy.

I have already become involved in directing research activities of Ms. Susan Compton, a doctoral candidate in this department in her studies of the function of aveolar type II epithelial cells in culture. This project is far from complete, but considerable progress has been made in the last several months.

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The primary aim has been to define conditions for the mass cultures of type II cells such that they continue to produce surfactant in vitro. To this end a number of variables have been investigated. At present the best success has been obtained by isolating type II cells from 3-4 day old neonatal rats by the procedure of Rounds (personnal communication) and establishing the cells as monolayer cultures with medium 199 with 10% fetal calf serum and dexamethasone (0.5 µg/ml). After 28 hours in culture the cells exhibit the characteristic lamellar bodies of type II cells in vivo. Autoradiographic studies have been initiated to establish the incorporation of 3H-palmitate into lamellar bodies per se, but these have not yet been completed. However, the growing cultures actively incorporate 3H-palmitate lecithin after culture for at least 3 weeks. Cells examined at 3 weeks of culture do not contain typical lamellar bodies but a high proportion have lipid-like inclusion bodies. The nature of these bodies and the time course of their appearance during culture are currently being studied.

To facilitate further studies of the culture conditons that would permit production of normal lamellar bodies in vitro we are now concentrating on light microscopic histochemical techniques for phospholipids that can be used to monitor more easily the effects of altered conditions on the cell populations.

As stated in Project 3 of the Program Project, I will also derive cultures of type II cells by the clonal isolation technique that has already been used for this purpose. However, if Ms. Compton's investigations succeed in establishing a procedure for obtaining mass cultures of type II cells these would be used for our experiments, as the clonal technique always carries the risk of selecting a clonal population that is aberrant in some respect as compared to the whole population in vivo.

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Addendum 2 (a)

MEMORANDUM TO: Dr. Paul Hamosh DATE: July 17, 1975

THROUGH: , Chairman, Department of

FROM: Clifton K. Himmelsbach, M.D.
Secretary, Research Committee

SUBJECT: Action on your request for approval of a proposal involving research on man

I am pleased to report that your proposal entitled,

"The Effect of Smoking on Lung Development"

was recommended for approval by the Research Committee on 5 / 15/ 75, and approved by the Executive Faculty on 5 / 23/ 75 with the understanding that:

1. the approval is contingent on your acceptance of the following modifications in your protocol:

--that parental consent be obtained since the subjects are to be High School students in the age range 12-14 years and provision be made to protect and identity of the participating subjects.

2. you will seek and obtain prior approval for any substantive modification in the approved protocol.

3. you will report promptly any unexpected or otherwise significant adverse effects encountered in the course of this study.

CKH/ah

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Addendum 2(b)

Georgetown University

CONSENT FOR CLINICAL STUDIES

AUTHORIZATION ON BEHALF OF A MINOR

I hereby authorize and agree to the participation of _____ (Born)_____ who is unable to provide personal consent, in a program of clinical studies expected to prove beneficial in the prevention of disease. The clinical studies will involve the following procedures:

1. Answer a questionnaire about vital statistics and smoking habits.
2. Blow into a device which measures the flow of air during exhalation

The purpose of the study is clear to me and I fully understand that the procedure is harmless.

_____

Signature of Parent or Guardian

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

July 17, 1975

Grant application No. 1045

CANCER

To: The committee comprising Drs. Feldman, Gardner, Huebner and Jacobson

Subject: Herbert B. Herscowitz, Ph.D., Georgetown University, D.C.
New application No. 1045
"Effect of Cigarette Smoke Exposure on Developmental, Cellular and Molecular Aspects of the Immune Response"

History

A three year plan, entitle "The Role of the Macrophage in the Immune Response: Effect of Tobacco Products on Macrophage Function", submitted by Herscowitz was approved by the SAB in October 1972. The second renewal (Grant No. 877R2) was approved by the SAB in October 1974 at a level of $25,515 for the period January 1, 1975 through September 30, 1975 when this grant terminates.

The new application (No. 1045) is a result of the success of the studies carried out in the three year period supported by Grant No. 877.

Request

Application No. 1045 requests $46,675 for the first year of a three year study: estimates for the second and third years are $47,000 and $50,910, respectively.

Documents submitted (attached)

1. Application dated June 30, 1975 (28 pages).
2. C.V.s of Drs. Herscowitz and Rabinowitz (9 pages)
3. Progress Report No. 3 (for Grant No. 877 covering period June 1, 1974 - May 31, 1975 (33 pages including Tables).

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4. (a) First page of a reprint with CTR acknowledgement.

(b) Abstract and summary of two manuscripts submitted to Journal of Immunology.

(c) First page of manuscript accepted by Prostaglandins.

(d) Abstract of paper given at FASEB meetings (1975).

N.B. Full copies of 4(a), (b) and (c) will be sent on request.

D.S.

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atts.

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THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED JUL 3 1975

Application for Research Grant
(Use extra pages as needed)

Date June 30, 1975

1. Principal Investigator (give title and degrees):

Herbert B. Herscowitz, Ph.D.
Assistant Professor of Microbiology

2. Institution & address:

Schools of Medicine and Dentistry
Georgetown University
3900 Reservoir Road, N.W.
Washington, D.C. 20007

3. Department(s) where research will be done or collaboration provided:

Department of Microbiology
Department of Pathology

4. Short title of study:

"Effect of cigarette smoke exposure on developmental, cellular and molecular aspects of the immune response."

5. Proposed starting date: January 1, 1976

6. Estimated time to complete: Three years

7. Brief description of specific research aims:

The adaptive immune response has evolved to an extremely refined state in the mammal where it serves a protective function against invasion by potentially pathogenic microorganisms and possibly even more important, to perform a surveillance function by keeping continuously arising malignant cell clones under control. Immunological defects, whether congenital or acquired, result in a susceptible host. The lung of the healthy individual is constantly being exposed to potential insult, which is handled successfully. One such environmental insult on the lung is cigarette smoke. Chronic exposure to cigarette smoke or its components may result in an altered immune response which will ultimately affect the host's health.

The major objective of this research proposal is to further our understanding of the effects of cigarette smoke on the immune response. We will determine the effects of acute and chronic smoke exposure on the functional capabilities of the three principal cell types involved in the immune response; B-cells, T-cells and macrophages. It is becoming increasingly apparent that adult disease is the culmination of a lifetime of insult. In this regard we will study the effects of cigarette smoke on maturational aspects of the immune response in an attempt to define factors which may identify a population at risk to increased frequency of infection or malignancy.

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8. Brief statement of working hypothesis:

It is postulated that the immune system serves a surveillance function for control of proliferation of malignant cell clones. Previous studies have shown that patients displaying an altered immune response, either congenital or acquired, are subject to the development of frequent infections and to various forms of malignancy. The alveolar macrophage plays a very significant role in host defense against foreign materials. Macrophages appear to be involved in both the efferent and afferent arms of the immune response to certain antigens, those complex materials which also require T-cell cooperation. It has been demonstrated that interference with macrophage function results in suppression of immune responsiveness. Our previous studies (see Progress Report #3) have shown that cigarette smoke inhibits humoral immune responses. The present studies will delineate the cellular and molecular mechanisms of the effects of cigarette smoke on the immune response.

9. Details of experimental design and procedures (append extra pages as necessary)

See attached Research Plan.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Laboratory space currently available to the Principal Investigator consists of one laboratory of approximately 750 sq. ft., including an office, a post-doctoral laboratory of approximately 300 sq. ft., a small animal room of 200 sq. ft. and temporary space in a presently unused laboratory for operation of the Walton Mark II Smoke Machine. My laboratory is equipped with refrigerated centrifuges, automatic gamma spectrometer, fraction collectors, pH meter, spectrophotometer, $CO_2$ incubator, phase contrast and fluorescence microscopes, electrophoresis equipment, columns for chromatography, facilities for handling of radioisotopes, glassware and reagents. Within the Department of Microbiology are a photographic darkroom, cold and warm rooms, media preparation and dishwashing facilities, amino acid analyzer, recording spectrophotometer, ultracentrifuges, electron microscope. Also available to me through participation in the Pulmonary Program Project is a scanning electron microscope. The Department of Pathology is well-equipped to carry out the processing of tissue specimens for microscopic examination.

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

See attached.

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

See attached.

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14. First year budget:

| A. Salaries (give names or state "to be recruited") | % time | Amount |
|---|---|---|
| Professional (give % time of investigator(s) even if no salary requested) | | |
| Herbert B. Herscowitz, Ph.D. | 50% | $4,431 + 817* = $5,248 |
| Max Robinowitz, M.D. | 10% | - 0 - |
| Technical | | |
| Ruth B. Cooper | 100% | $11,000 + 2,200* = $13,200 |
| To be recruited - 1/2 time lab aide (dishwasher, animal caretaker) | 50% | 3,000 + 600* = 3,600 |
| * Fringe Benefits | | |
| Sub-Total for A | | $ 22,048 |

| B. Consumable supplies (by major categories) | |
|---|---|
| 1. Glassware | 1,000 |
| 2. Disposables (plasticware for culture) | 2,500 |
| 3. Tissue culture media | 2,000 |
| 4. Chemicals, isotopes, assay materials | 1,500 |
| 5. Animals (rabbits and mice) | 2,500 |
| 6. Miscellaneous | 500 |
| Sub-Total for B | $ 10,000 |

| C. Other expenses (itemize) | |
|---|---|
| 1. Animal care | 4,000 |
| 2. Travel | 500 |
| 3. Publication | 500 |
| 4. Equipment maintenance (service policies) | 800 |
| 5. Pathology service | 1,000 |
| Sub-Total for C | $ 6,800 |
| Running Total of A + B + C | $ 38,848 |

| D. Permanent equipment (itemize) | |
|---|---|
| Miscellaneous small lab replacement | 2,000 |
| Sub-Total for D | $ 2,000 |
| E. Indirect costs (15% of A+B+C) | E 5,827 |
| Total request | $ 46,675 |

15. Estimated future requirements:

| | Salaries | Consumable Suppl. | Other Expenses | Permanent Equip. | Indirect Costs | Total |
|---|---|---|---|---|---|---|
| Year 2 | 24,000 | 9,000 | 7,000 | 1,000 | 6,000 | 47,000 |
| Year 3 | 26,400 | 10,000 | 7,000 | 1,000 | 6,510 | 50,910 |

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|---|---|---|---|
| 1. Immunological Function of Alveolar Macrophages | NIH-HL/AI-16748-02 | $43,792 (Direct) | May, 1975-April, 1976 |
| 2. Preparation of Antibodies Against Prostaglandins | Coleman Associates Contract | 1,600 | February, 1975-September, 1975 |

PENDING OR PLANNED

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|---|---|---|---|
| None | | | |

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal Investigator

Typed Name Herbert B. Herscowitz

Signature Herbert B. Herscowitz Date June 30, 197

Telephone 202 (Area Code) 625-7602 (Number) (Extension)

Checks payable to

Mr. George R. Houston, Jr., Treasurer

Mailing address for checks

Georgetown University
37th and O Streets, N.W.
Washington, D.C. 20057

Responsible officer of institution

Typed Name Sam A. Kimble

Title Administrator of Sponsored Programs

Signature Sam A. Kimble Date June 30, 197

Telephone 202 (Area Code) 625-3151 (Number) (Extension)

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Budget Justification

1. Duration, Reason for Proposed Period of Support

Support is requested for a three year study. The first year will be devoted to a continuation of the studies of the effect of chronic smoke exposure on immune function. The second and third years will study the cellular and molecular mechanisms along with developmental aspects of immune response.

2. First Year Budget

a. Salaries. In accordance with the policy of Georgetown University, I am requesting that 20% of my salary be paid from this grant. A full-time laboratory technician will be required to participate in this program. I am requesting funds for a 1/2 time laboratory aide to help in the preparation of glassware, sterilization and care of mice used in these studies. There is presently such a person working in my laboratory and being paid from other funds. However, due to expanded effort, the work is too much for only 1/2 time employment. Therefore, contributions from both grants will enable us to have full-time assistance. Also included in these figures are fringe benefits set by the University at 16.7% and 20.0% for 1974-1975 and 1975-1976, respectively.

b. Supplies. These amounts are self-explanatory.

c. Animals. The quality of experimental animals determines the high budget request. In addition, animal costs have been routinely increased, with 10% service charges added on by the Vivarium.

d. Animal Care. The University Animal Care Facility provides us with quarters and care by trained personnel. The costs of these services has risen tremendously over the past years and are scheduled to be increased as of July 1, 1975 again.

e. Travel. Funds are requested for the Principal Investigator to attend one meeting each year which will have relevance to the proposed research. This meeting is usually the FASEB meeting of the American Association of Immunologists which will be held in California next year.

f. Pathology Service. Funds in the amount of $1,000 are requested to cover expenses of Dr. Robinowitz for the pathological examination of the lung material. This amount includes the cost of chemicals, reagents, stains, slides and preparation.

3. Budget for Future Years

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a. Salaries. We are planning to retain the same personnel with an anticipated 10% increase in salary which was proposed by University regulations.

b. Other Items. There should be no changes in these amounts which are necessary to meet the inflationary rise of scientific supplies.

Source: https://www.industrydocuments.ucsf.edu/docs/xhdm0000

Research Plan

A. Background:

It is the mammalian lung that effects the transfer of oxygen by exposing almost the entire volume of circulating blood to the environment. Whereas the relationship between internal and external environments supplies the oxygen necessary for an aerobic existence, it also renders the lung vulnerable to insult by materials carried in ambient air. There is an increasingly expanding body of convincing scientific evidence which suggests that most, if not all pulmonary disease can be related to the inhalation of infectious agents or irritating particulate matter. However, there exists a tremendous gap between scientific knowledge and public attitude in the case of cigarette smoking. While there is as of yet no hard and firm evidence to establish a direct causal relationship, there has been an almost epidemic increase in carcinoma of the lung and chronic obstructive lung disease associated with increased cigarette smoking. There have been numerous retrospective and prospective studies that have shown that the risk of a smoker dying from lung cancer to be as high as 14 times that of a nonsmoker. Cigarette smoking is also closely related to the clinical syndromy commonly grouped together as chronic obstructive lung disease. One such clinical disease, emphysema, is characterized by destruction of alveolar tissue with enlargement of air space distal to the terminal bronchiole, resulting in progressive shortness of breath. Another condition, chronic bronchitis, characterized by hypertrophy of bronchial mucous glands which leads to production of excessive mucus resulting in a chronic productive cough. Carcinoma of the lung and chronic obstructive lung disease are clinically distinct entities, however, they may represent the final stage of continuous irritation.

The effect of cigarette smoking on the human lung was identified during routine autopsy studies performed on healthy young victims of sudden and unexpected death. Niewoehner et al. (1) studied the lungs of 39 such individuals who averaged 25 years of age. Nineteen of the 39 were smokers and 12 of the 19 had either productive coughs or frequent colds. Of the 20 nonsmokers, only 3 had evidence of any respiratory symptoms. Loss of bronchial epithelium and evidence of bronchial wall inflammation were common in the smokers. The most striking abnormality was respiratory bronchiolitis, a lesion of the small airways found in all of the smokers, but also in 5 of the nonsmokers. Clusters of brown pigment-containing macrophages were found in the first and second respiratory bronchioles; edema, fibrosis, and epithelial hyperplasia were seen in the thin walls of these small airways.

There is some evidence which supports the belief that the irritant potential of cigarette smoke first manifests itself as bronchitis and emphysema and ultimately leads to neoplasia. The early work of Auerbach et al. (2) in a detailed blind study of the tracheobronchial epithelium of 456 men and 302 women showed that loss of cilia, basal cell hyperplasia and appearance of cells with atypical nuclei were more common in smokers and increased in proportion to the number of cigarettes smoked. Extensive atypical changes were commonly seen in patients smoking more than 2 packs of cigarettes per day. They also found in one patient, a squamous cell carcinoma in one area together with multiple areas of atypical basal cell hyperplasia at a distance from the carcinoma. However, one should not rule out the possibility that different components of cigarette smoke are responsible for the inflammatory and neoplastic changes observed.

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The mechanisms by which the lung defends itself against the effects of infectious agents, toxic substances, and allergens are complex and still incompletely understood. There is evidence that both humoral and cell-mediated immune responses play a protective role in the lung (3-8). Immunologic damage also occurs in the lung. This is associated with the host's inflammatory response to antigenic material from microorganisms or to proteinaceous material emanating from insects or animal matter, such as pigeon excretia, animal dust, etc., or to reactive chemicals which may function as haptens.

Since any one segment of the respiratory tract differs from others in terms of vascularization, lymphatic drainage, distribution of lymphoid tissue, availability of macrophages, and microanatomy of the mucous membrane, it can be anticipated that the site of deposition of immunogenic material in the respiratory tract will be one of the major factors determining the qualitative and quantitative aspects of the subsequent immune response, and that the site will also be of major pathogenetic significance in the development of immunopathological diseases of the respiratory tract. Creasia et al. (9) showed that upon intratracheal injection of particulate material into small rodents the primary site of deposition was the distal lung. Nettesheim and Williams (7) employed this observation to determine whether antigens that are allowed to penetrate to the alveolar parenchyma are capable of effectively triggering an immune response and where this immune response is located. Using respiratory-disease-free mice, they showed that particulate antigens (RBC) were ineffective in triggering a humoral immune response when deposited in the deep lung. However, when larger amounts were applied, an immune response was observed and the spleen appeared to be the major location of most of the antibody-forming cells. In addition, the spleen also contained primed memory cells which responded to secondary antigenic stimulus. This was shown by experiments in which either the numbers of antibody-forming cells were enumerated in the lungs, lymph nodes and spleens of intratracheally-exposed animals or by experiments where the spleen was removed at various times before or after antigenic challenge. Their studies also demonstrated that once a primary antibody response had been successfully induced via the respiratory tract, local as well as systemic immunity developed, that is, an anamnestic response could be initiated via the intratracheal, as well as via the intravenous route, accompanied by a massive influx of lymphoid elements into the lungs in both cases. Hiramoto and Hamlin (10) previously showed that antibody-forming cells appeared in the lung during an anamnestic response in which both primary and secondary antigenic challenge were given intravenously. Nettesheim and Williams (7) concluded that nontoxic, particulate antigens deposited in the alveolar parenchyma are readily eliminated or rendered non-immunogenic and that only a small proportion of the immunogen is capable of entering the blood stream, probably at the level of alveolar capillaries and initiates a systemic immune response. These findings are in contrast to the studies of Holub and Hauser (11) who reported a predominantly local antibody response by lung alveolar histiocytes after intratracheal instillation of antigen and the studies of Johnson and Waldman (3) in which it was shown that local introduction of antigen into the respiratory tract resulted in an optimal local pulmonary response with little or relatively less evidence of systemic responses; the converse was also true. Of further interest were their studies in which mice were exposed to killed Bordetella pertusis organisms or to live Sendai virus prior to intratracheal or intravenous stimulation with the hetero-

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logous RBC. They observed a marked inflammatory response as a consequence of the additional microbial agents which was accompanied by an enhanced antibody response to SRBC when the antigen was administered intratracheally. These experiments suggested that respiratory infections might play an important role as "conditioners" or "adjuvants" in the induction of the immune response in the deep lung. Finally, their studies also demonstrated secondary responses for pulmonary cell-mediated immunity which suggests the probability of a capacity for memory by pulmonary T-lymphocytes.

One of the major cell types of the alveolus is the alveolar macrophage. This mononuclear cell is derived from a bone marrow precursor but undergoes differentiation in the lung presumably to adapt to the high oxygen environment of the alveolus. This cell is the principal macrophage of the lung and is responsible for the uptake and removal of inhaled particles (12). Thus it acts to protect the respiratory membrane from the tissue-damaging properties of inhaled particles and also may act as a transport which out of the lung for foreign materials. The intrapulmonary processing of foreign particles deposited on the respiratory membrane depends on the primary and secondary mechanisms directed at the physical, chemical and biological properties of the particle which are associated with its pathogenic potential. Primary mechanisms include the coating actions of secretory materials, chemical interactions with oxidant gases, solubilization of salts and phagocytic ingestion by resident alveolar macrophages. The primary particle processing mechanisms are enhanced by secretory, humoral and cellular-immune mechanisms resulting from previous exposure. Secondary cellular and humoral defense mechanisms are brought into play when the primary systems fails to process adequately the inhaled particle. This complex set of mechanisms provides many opportunities for defective function in particle uptake or intracellular processing phases of phagocytosis, in the subsequent immune responses, and in protective mechanisms against the proteolytic enzymes released in the inflammatory response. For example certain particulate materials such as silica or asbestos may induce direct lysozomal damage, cell death, and inflammation (13). Environmental gases inhibit cell enzymes; cigarette smoke inhibits glyceraldehyde dehydrogenase, anaerobic glycolysis and particle uptake (14). Such environmentally-induced defects in macrophage handling of foreign particles may have their ultimate effect on the ability of the host to mount an immune response.

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In recent years, a great deal of information has been gathered in relation to the cellular components of the immune response. In mammals, like in avian species, there are two primary lymphoid compartments. The thymus, which has an influence on the cells (T-cell) that participate in cell-mediated immunity and the analogue of the bursa, which has an influence on the cells (B-cells) that synthesize antibody. In addition to their separate functions it has been shown that the ability of a B-cell which has been exposed to antigen to initiate antibody production depends for many, but not all, antigens on the concomitant activity of specifically stimulated T-cells. These T-cells can exert a regulatory influence on several B-cell functions: (a) the switch from IgM to IgG antibody synthesis; (b) selection of clones producing antibody of high affinity; (c) suppression of B-cell function under certain circumstances (15). There is also an impressive body of literature which implicates a third cell type in both the efferent and afferent limbs of the immune response. This cell type, referred to as an accessory cell, has enjoyed varing degrees of favor among immunologists in regard to cyclical feelings of its importance in the immune response. There are numerous reviews which have been devoted to the function of macrophages, especially their role in the immune response (16-19).

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By definition, the basic functional property of a macrophage is its ability to engulf foreign materials. Macrophages take up materials essentially by two similar processes, phagocytosis (particulate materials) and pinocytosis (soluble matter). Both processes are initiated by the interaction of the foreign material with the cell membrane (20). The material, after entry into the cell, becomes surrounded by a membrane which eventually coalesces to form the phagosome. The phagosome moves to the interior of the cell where it subsequently fuses with a preformed lysosome to form a phagolysosome (21). The hydrolytic enzymes of this secondary lysosome catabolize the ingested material and for many substances, the endocytic function of the macrophage is fulfilled. It is this product of endocytosis that has intrigued immunologists, for this may be the initial stage of antibody formation.

The relevance of phagocytosis to immune processes of host protection is still somewhat unclear. While some suggest that there is no correlation between susceptibility to phagocytosis and immunogenicity (22) others have shown that some of the phagocytized antigen remains associated with the macrophage surface and is more immunogenic than free antigen alone (23). Evidence for the role of the macrophage in humoral responses was provided by numerous in vivo studies using irradiated mice (24), reticuloendothelial blockade (25) and anti-macrophage serum (26-28). The essentiality of macrophage participation in the primary in vitro immune response was shown by Mosier (29). In mouse spleen cultures, antibody production appeared to involve both the phagocytosis of antigen by a cell population capable of adhering to glass (macrophage-rich) and antibody production by a non-adherent cell population (lymphocyte-rich). Later, the non-adherent cell population was further resolved into its T and B cell components (30). Several possible functions have been assigned to the macrophage in its participation in the immune response: (a) antigen processing; (b) antigen presentation due to surface-bound components; (c) providing the necessary microenvironment for lymphoid cell survival; (d) mediation of the signal for antibody production by B-cells. An intriguing model of antibody formation recently proposed by Feldmann (31) employing the three functional cell types does not preclude any of the above mentioned roles for the macrophage.

The literature discussed above comprises only a small proportion of that which exists regarding the role of the macrophage in the induction of the humoral immune response. Further, the literature regarding the role of the macrophage in cell-mediated immunity is almost as voluminous and has recently been reviewed by Nelson (32). It soon becomes apparent that there is no consistent agreement among investigators as to the precise role of macrophages in immune responses and a great deal is yet to be done to further our understanding. 1003545778

To return to the main stream of purpose, the alveolar macrophage system constitutes an important cellular defense mechanism in the lung, by virtue of its role in removing foreign substances. It is also possible that these cells cooperate with lymphocytes in situ in the lung or transfer antigen to lymphocytes in regional lymphoid organs. Consequently, any environmental factors that interfere with the function of alveolar macrophages may be considered to be of potential danger to the immune capabilities of the host. In recent years there has been a significant amount of interest in the effect of tobacco smoke on macrophage function. The exposure of alveolar macrophages to tobacco smoke or its components has been shown to induce a number of metabolic and functional changes in these cells. Alterations have been demonstrated in the resting rate of glucose oxidation (33), protein synthesis (34), the activity of various enzymes (35-36), ultrastructure (33,37), cell growth and nucleic acid synthesis in vitro

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(38-39), and phagocytic ability (40-41). In addition, recent reports suggest that cigarette smoke or components thereof have an adverse effect on the immune response. Holt and Keast (42) studying the acute and chronic effects of cigarette smoke on mice have demonstrated significant death of cells of the immune series both in vivo and in vitro following acute smoke exposure. Chronic exposure to smoke on the other hand resulted in increased numbers of macrophages and lymphocytes in the lungs of experimental animals. Finklea et al. (43) have shown that young adult cigarette smokers had significantly lower concentrations of antibody to influenza virus $A_2$ after either natural infection or vaccination as compared to a similar group of nonsmokers. Roszman and Rogers (44) demonstrated that there was a dose-dependent suppression of the in vitro anamnestic response of rabbit lymph node cells to SRBC by nicotine and the water-soluble fraction of cigarette smoke. Suppression could be achieved after exposure of the cultures to either agent for a period of 2 hours before antigenic challenge, thereby suggesting that these materials induce a rapid and irreversible effect on lymphoid cells. Using the C57 Bl mouse as a model, Thomas et al. (45) showed that mice receiving 26 weeks of exposure to cigarette smoke had decreased primary and secondary immune responses in all organs examined after intratracheal instillation of SRBC. The primary response in the lung was completely suppressed whereas the secondary response was delayed. Primary responses in mediastinal and cervical lymph nodes and spleen were short-lived while secondary responses in these organs were delayed. In an additional study, the same authors (46) exposed mice to cigarette smoke for up to 42 weeks. In mice exposed to smoke only during the week following immunization, the response was not impaired in any of the lymphoid organs examined. However, a decrease in the primary response in the lungs was observed in mice exposed to smoke for 9 days prior to antigen administration. The responses found in spleen during the initial 120 days of exposure were erratic. However, after 294 days of exposure the response in spleen was depressed in all animals. The lymph node response was consistently first enhanced and then depressed by cigarette smoke exposure. Esber et al. (47) showed that inhalation of whole cigarette smoke or its vapor phase impaired the hemagglutinin response of mice injected with SRBC. An immunosuppressive effect was observed in animals exposed to whole smoke or vapor phase for seven days before or two days after administration of antigen. This effect was apparently temporary since there was recovery of immune responsiveness about 14 days after exposure. It was further found that the early or inductive phase of both primary and secondary responses rather than the late or productive phase was affected by cigarette smoke. This suggests that the suppressive effect of cigarette smoke may be functioning at the level of the macrophage. A similar recovery from the immunosuppressive effects of cigarette smoke has been reported by others (48). In an additional study, Thomas et al. (49) showed that there were only minor differences in immune responses of animals exposed to whole smoke and challenged with antigen by a systemic route rather than intratracheally. This appears to be in opposition to the results of Esber et al. (47) described above.

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Studies of the effects of cigarette smoke on cellular immune function have also recently appeared. Rylander (50) showed that guinea pigs used in four and eight-week inhalation experiments had increased numbers of macrophages and leukocytes in the lung. Smoke that had been passed through Cambridge filter did not cause the effects. Thomas et al. (51) exposed mice daily to fresh cigarette smoke for up to 35 weeks and evaluated cell-mediated immunity as measured by Phytohemagglutinin responsiveness of lymphocytes from various organs. They observed an initial enhancement of PHA responsiveness in lymphocytes from regional lymph

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nodes and peripheral blood but by the 35th week of exposure the response was markedly suppressed. On the other hand, Suciu-Foca et al. (52) and Whitehead et al. (53) demonstrated that there were no differences in PHA responsiveness in lymphocytes obtained from smokers compared to age-matched non-smoking controls. The latter study also showed that the addition of nicotine to cultures of peripheral human lymphocytes had no effect on PHA-responsiveness. These studies are in contrast to the study of Desplaces et al. (54) who found tobacco smoke inhibited lymphocyte by PHA but stimulated lymphocytes in the absence of PHA. The studies of Warr and Martin (55) showed that MIF reduced the migration of alveolar macrophages from nonsmokers but was ineffective in preventing migration of alveolar macrophages from smokers. Migration of macrophages from nonsmokers with delayed type hypersensitivity skin reactions to Candida was inhibited when antigen was present in the medium. Antigen did not inhibit macrophages who lacked delayed hypersensitivity to the antigen or macrophages from cigarette smokers. Finally, Roszman et al. (56) showed that nicotine or water soluble fraction of whole cigarette smoke inhibited transformation of rabbit peripheral blood lymphocytes stimulated with anti-rabbit Fab (B cell mitogen) and concanavalin A (a T cell mitogen). These results deserve further investigation in terms of the role of macrophages in these responses.

Although the above described studies represent a vast refinement of experimental design over those previously reported it is quite obvious that there is a lack of agreement of experimental results. Whereas one group demonstrates an irreversible effect of cigarette smoke, others point toward reversibility. Whereas some find a suppressive effect on systemic immunity, others limit the effect to the lung. Where some restrict the inhibitory effect to particulate matter in whole smoke others find it with the gas phase. Whereas some find an effect on cell-mediated immune function others do not. All of these differences may be due either in total or in part to the differences in cigarettes used, experimental animal, state of health of animal and conditions used for smoke exposure. In order to generate meaningful results these conditions must be cautiously defined and clearly stated so that results can be compared.

B. Rationale and Working Hypothesis:

It is generally accepted that the adaptive immune response evolved as a general vertebrate protective mechanism to eliminate "non-self" components (57). Burnet suggested that the immune system functions as a surveillance mechanisms to aid the individual against life-threatening challenge. The surveillance concept has been suggested as a defense in human cancer. In simplest terms, surveillance assumes that tumor cell clones emerge continuously during the life of an individual, and that effective mechanisms exist by which the foreigness (tumor clone) is recognized, attacked and destroyed. This can be explained on an immunological basis. There is sufficient evidence to indicate that in the process of transformation to a neoplastic condition, the cell acquires which were not present on the normal parent cell, thus the cell is no longer recognized as self and as a consequence is rendered susceptible to immune attack on the basis of its newly recognized antigenic makeup. One prediction of this theory is that individuals who have immune deficiency, either congenital or acquired, should be subject to a higher incidence of cancer. Indeed, the evidence of Good and Finstad (58) indicates that patients who have congenital immunological defects and patients who have been immunosuppressed following organ

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transplant develop neoplasms with a greatly increased frequency. Further, in man, the incidence of cancer greatly increases with ensuing age. It has thus been suggested that the development of cancer is associated with a progressive inability to eliminate foreigness which is related to a decrease in efficiency of the immunological defense mechanisms (59). It should be noted that most chemical carcinogens are also immunosuppressive (60) and that many, if not most, viruses known to cause cancer in animals can also suppress the host's immunological reactivity. Thus it would appear that any alteration of the immunological defense mechanism may contribute to the establishment of malignant cells and the eventual demise of the host.

It is apparent that the cells of the reticuloendothelial system are actively involved in maintaining the health of the individual. Numerous studies suggest that macrophages are involved in the induction of both the humoral and cell mediated immune response, probably at the level of antigen processing. There is evidence to indicate that blockade of RES function results in an inhibition of immune responsiveness. Several studies have shown that tobacco products interfere with the phagocytic capabilities of the macrophage as well as with the host's immune response. This research proposal is designed to determine the cellular and molecular mechanisms, involved in the immunosuppressive effect of cigarette smoke.

C. Specific Aims:

The specific aims of this research proposal are to obtain an understanding of the effects of cigarette smoke on the immune response. We are interested in determining the cell type affected and the molecular mechanisms involved. We will study the effects of cigarette smoke on the maturation of the immune response to determine potential populations at risk to increased infection or malignancy. Experiments will be devised to elaborate and evaluate the following parameters:

Year 1

1. To further define the immunosuppressive effect of cigarette smoke on the immune response of mice.
   a. To study the effect of chronic smoke exposure on humoral antibody production.
      (1) To compare Kentucky Reference cigarettes (1A1 and 1R1) in these studies.
      (2) Evaluate plaque-forming cells and serum antibody levels.
   b. To study the effect of chronic smoke exposure on correlates of cell-mediated immunity.
   c. To evaluate the immunosuppressive effect of the vapor phase of cigarette smoke on humoral and cell-mediated immunity.
      (1) To compare 1A1 and 1R1 cigarettes
      (2) To compare acute and chronic exposure to smoke.

Year 2

1. To determine the effect of cigarette smoke on the individual cell types involved in the immune response.
   a. To study the effect of acute and chronic smoke exposure on macrophage function.
      (1) To determine the effect of *in vitro* and *in vivo* smoke exposure on phagocytic and antigen-processing capabilities.
   b. To study the effect of acute and chronic smoke exposure on B-cell function in the absence of T cell regulatory effects.

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(1) To employ the congenitally athymic mouse in studies of immune function.

c. To study the effect of smoke exposure on T-cell function.

d. To determine the effect of cigarette smoke on macromolecular synthesis in B cell, T cells and macrophages.

Year 3

1. To determine the effect of cigarette smoke on developmental aspects of the immune response.

a. To study the long term effects of cigarette smoking of pregnant mice on the immune function of their progeny.

b. To study the effects of cigarette smoke on immune function of animals of different ages.

(1) To determine if neonatal exposure to cigarette smoke has any long term effects on the quality or quantity of immune responsiveness.

All throughout these studies, the lungs from mice exposed to the various smoke exposure protocols will be supplied to Dr. M. Robinowitz of the Pathology Department who will evaluate them for intercurrent microbial infection and for smoke-induced pathological changes. Having materials from animals involved in clearly defined and controlled smoke exposure protocols will enhance our understanding of potential pathology induced by cigarette smoke.

D. Experimental Design and Procedures:

1. General Outline

It is becoming increasingly clear that requirement for collaboration among different cell types in immune responses is a function of the physical and chemical structure of the antigen. The chemically complex materials, that is, those substances containing a small number of diverse antigenic determinants appear to require the collaborative interaction of T and B cells for antibody production. On the other hand, those antigens which contain a large number of repetitive chemical structures appear to stimulate B cells directly in the absence of T cell help. In a like manner, the requirement for macrophage participation in immune responses also appears to be based on antigen structure. The complex, particulate antigens appear to require some interaction with macrophages, either for processing or presentation to the appropriate immunocompetent cell type. The less complex materials appear to be independent of macrophage handling. Thus one could speculate that the T-dependent antigens also require macrophage participation in the initiation of immune responses, whereas T-independent antigens are also macrophage-independent.

Our previous results (see Progress Report #3) have shown that acute exposure of mice to smoke from Kentucky Reference 1R1 cigarettes caused marked suppression of the appearance of plaque forming cells in the spleen. Our working hypothesis is based on convincing evidence which suggests that macrophages contribute to the initiation of immune responses at the level of antigen processing to many, but not all antigens. Further, it has been suggested that interference with the phagocytic function of the macrophage results in an altered immune response. Therefore, based on this evidence the most likely target cell for the immunosuppressive effect of cigarette smoke would be the macrophage. Although this cell type has been implicated there has been no rigorous demonstration of the interference. One of our major goals therefore will be to determine the effects of cigarette smoke at the cellular and molecular level. Our laboratory

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has the animal systems, techniques and reagents available to undertake such a study. For example, our anti-macrophage serum can be used to obtain populations of B and T cells devoid of macrophages, whereas our anti-plasma cell serum can be used to obtain populations of T cells and macrophages. Using these reagents in combination in the athymic mouse system we can obtain "pure" populations of B cells and macrophages. One test of the hypothesis that T-independent antigens are also macrophage independent and a possible means of determining the cells affected would be to employ nude mice in smoke exposure protocols. At this time, I would predict that such animals would be refractory to the immunosuppressive effects of cigarette smoke if macrophages were the target cells and were not required for the immune response to T-independent antigens. We can also use these isolated cell types to study the in vivo and in vitro effects of cigarette smoke on response to mitogens and phagocytic capabilities.

Another major emphasis of this proposal is directed toward an understanding of the effects of cigarette smoke on developmental aspects of the immune response. Newborn mice fail to produce antibodies to SRBC (61) and newborn rats will accept allogenic skin grafts (62). Newborn rats have been shown to lack the follicular and medullary antigen-trapping structure characteristic of adult animals. Further, there is an apparent absence of functional macrophages in newborn animals. Moreover, the transfer of macrophages from adult donors into newborn animals has resulted in an increased immune response thereby suggesting that immunological immaturity may be due, at least in part, to an inability of the neonate to handle antigens, thus ascribing this defect to immaturity of macrophages (61,63-64). As part of our Pulmonary Program Project, my laboratory is interested in the immunological functions of alveolar macrophages, specifically their ability to serve an accessory function in humoral responses. One aspect that will be studied in collaboration with Dr. J. Bellanti is the maturation of immunological function of alveolar macrophages. To extend this further, the present protocol will study the effects of cigarette smoke on the maturation of immune responsiveness. We are interested in determining the effect of cigarette smoke on the immune responsiveness of progeny obtained from smoked-pregnant mice.

2. General Procedures

a. Animals

Two animal systems will be used in these studies. Rabbits will be used for studies of the in vitro anamnestic response and as a source of alveolar macrophages for the following reasons: the lung is the prime target of exposure to tobacco products, the rabbit is of sufficient size to yield a workable number of cells, and little is known concerning the contribution of alveolar macrophages to the immune response. Balb/c mice will be used for continued in vivo studies of the effect of cigarette smoking on the immune response, in vitro studies, mitogen studies, developmental studies and as a source of macrophages.

(1) Isolation of Macrophages:

The method of Myrvik et al. (65) will be employed to obtain unstimulated rabbit alveolar macrophages. Briefly, rabbits will be sacrificed by injection of a lethal dose of Diabutal (Diamond Labs, Des Moines, Iowa), the chest cavity opened aseptically and the trachea closed by means of a sterile

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hemostat. The trachea will be transected above the clamp and the lungs, heart and trachea will be dissected out. A volume of 30 ml. of Hank's balanced salts solution will be injected into the trachea until the lungs are distended, the lungs massaged and the fluid drained into sterile centrifuge tubes. The cells will be recovered by centrifugation at 1,500 rpm for 15 minutes and resuspended in culture medium containing 20% normal rabbit serum. The cell suspension so obtained has been shown to be contaminated by less than 2% of other cell types.

Both stimulated and unstimulated peritoneal macrophages will be obtained from mice. For stimulated peritoneal exudate cells, mice will be injected with 3 ml. of fluid thioglycollate medium (Difco Labs, Detroit, Mich.), according to the procedure of Argyris (66). Three days later, the mice will be sacrificed by exposure to chloroform vapor and 3 ml. of BSS containing 30 units of heparin will be injected into the peritoneal cavity. After gentle massage of the peritoneum, the fluid will be collected and cells recovered by centrifugation as above. The cell pellet will be resuspended in culture medium containing 20% of new-born calf serum. Unstimulated peritoneal cells will be collected by a similar procedure except that fluid thioglycollate will not be injected. Alveolar macrophages will also be obtained from mice by a modification of the Myrvik procedure, or as described by Bennett (67).

b. Antigens

Keyhole limpet hemocyanin (KLH) which is a potent immunogen, inducing high concentrations of antibody, evoking high levels of immunological memory, and involving a large number of cells in the synthesis of antibody (68) will be used as the antigen. KLH obtained as an ammonium sulfate slurry (Pacific Bio-Marine Supply Co., Venice, Calif.) will be purified according to the procedure developed in our laboratory (69). Briefly, after extensive dialysis, the material will be concentrated by ultrafiltration using an XM-100 membrane (Amicon Corp., Lexington, Mass.). The KLH will be fractionated on a column of DEAE-cellulose employing a linear salt gradient of 0.05 M NaCl - 0.05 M $PO_4$, pH 6.7 to 0.4 M NaCl - 0.05 M NaCl - 0.05 M $PO_4$, pH 6.7. The first peak eluted from the column will be concentrated by ultrafiltration, dialyzed and fractionated on a column of Bio-gel A 5 m (Bio-Rad Labs, Richmond, Calif.). The first peak, which is the purified KLH, shows a single component as determined by the following criteria: double diffusion in agar, immunoelectrophoresis, analytical ultracentrifugation, and disc electrophoresis. This material will be adjusted to a final concentration of 2 mg/ml, sterilized by Millipore filtration and stored at 4°C until used.

Sheep erythrocytes will be obtained from Microbiological Associates, Besthesda, Md., as a 20% suspension in Alsever's solution. The cells will be washed 3 times in cold phosphate buffered saline, pH 7.2, and resuspended in the same buffer at the appropriate concentration.

The thymus-independent antigens, dextran isolated from Leuconostoe mesenteroides B-1355 as described by Proctor (70) and lipopolysaccharide (Difco Labs, Detroit, Mich.) will also be used in these studies.

c. Tissue culture methods

(1) In vitro primary immune responses

Spleen-cell cultures will be prepared from inbred mouse strains



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as described by Mishell and Dutton (71). Cell suspensions will be diluted to the desired concentration in monolayer type MEM containing Hank's salts supplemented with sodium pyruvate, amino acids, vitamins, glutamine and 10% fetal calf serum, all obtained from Grand Island Biological Co., Grand Island, N.Y. The cultures will be distributed in Petri dishes at varying cell concentrations and incubated at 37°C in an atmosphere of 10% $CO_2$ and 83% $N_2$ on a rocker platform. The culture dishes will receive supplemental nutritional mixture and fetal calf serum daily as described (71).

(2) In vitro secondary response

Lymph nodes or spleens from rabbits previously primed with KLH in vivo will be surgically removed, trimmed of connective tissue and weighed. Cell suspensions will be prepared by disrupting the organ with stainless steel screens. Cells will be washed in 0.15 M PBS, pH 7.2, and adjusted to a final concentration of $2 \times 10^7$ nucleated cells/ml. Culture will be carried out in MEM as we have described previously (72). To detect newly synthesized antibody, $^{14}$C-leucine will be added to the culture medium and cells incubated at 37°C on a roller drum.

A portion of the cell suspensions will be stimulated with 100 µg of KLH while another portion will be incubated with BSS and will serve as control. After 24 hours, the antigen will be removed from cultures by sedimentation of the cells followed by washing to remove unbound antigen. The culture medium will be changed completely on alternate days. For the determination of newly synthesized antibody, the extracellular medium will be dialyzed extensively to remove unincorporated $^{14}$C-leucine and subjected to the assay procedure described below. For the determination of cells engaged in antibody formation, cell samples will be removed on various days and assayed by the plaque technique which will be described below.

d. Methods for evaluation of immune response

(1) Plaque assays

(a) Sheep erythrocytes

Cells releasing hemolysin for SRBC will be enumerated on the 4th or 5th day of culture by the PFC technique of Jerne and Nordin (73) as modified by Cunningham and Szenberg (74) for use on glass slides. Cells will be scraped from culture dishes and transferred to glass tubes where they will be dispersed. After determination of recovery and cell viability, 0.05 ml. of cells will be mixed with SRBC and complement and transferred to glass microscope slides. Plaques will be developed during a 1-2 hour incubation period at 37°C and counted. A similar procedure will be used for spleen cells from animals used for in vivo protocols.

(b) Keyhole limpet hemocyanin

Cells will be assayed for their ability to form hemolytic plaques against KLH-coupled RBC as described previously (75). Briefly, spleen cells at a concentration of $4 \times 10^6$/ml will be mixed with $4 \times 10^8$ SRBC/ml which have been conjugated with KLH using chronic chloride. After addition of guinea pig complement the mixtures will be added to each of 3 chambers formed on a glass slide and incubated at 37°C. Plaques will be enumerated by examining the slides under low magnification. To detect indirect plaques (IgG, PFC), an appropriate concentration of anti-IgG facilitating serum will be added to the cell mixture.

(c) Dextran, Lipopolysaccharide

Plaque assays for these antigens will be carried out according to the method of Proctor (70).

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(2) Radiolabeled protein and antibody assays

Newly synthesized extracellular proteins will be determined by precipitation of culture fluids with cold 5% TCA as previously described (72). De novo synthesized antibody will be measured by reaction of culture fluids with an insoluble immunoabsorbant as described by Self et al. (76) and modified by Herscowitz and Stylos (77). This method is an extremely sensitive and specific assay of antibody synthesis including antibody of low affinity.

(3) Hemagglutinating antibody

Serum antibody against SRBC will be assayed by standard hemagglutination using the Microtiter system (Cooke Engineering, Alexandria, Va.). Antibody against KLH, dextran or LPS will be measured by passive hemagglutination in the same system using methods described above for preparation of antigen-coated RBC.

e. Mitogen studies

Determination of the optimal concentration of mitogen (Phytohemagglutinin P, Difco Labs.; Concanavalin A, Miles Labs.; Lipopolysaccharide - Salmonella typhosa 0901, Difco Labs.) will be carried out according to the method of Peavy et al. (78). Brifefly, $3 \times 10^6$ cells are incubated in 0.8 ml of complete MEM containing 10% fetal calf serum, 2 mM glutamine, MEM nonessential amino acids, penicillin, streptomycin and fungizone and 37°C under 5% $CO_2$ in air for 48 hours in the presence of varying concentrations of the mitogen. After this period, 1 µCi of methyl-$^3$H-thymidine will be added and incubation continued for 24 hours. Cells will be harvested by centrifugation, washed in cold 0.15M NaCl, twice in cold 5% TCA and once in cold methanol. The precipitates are then air dried, solubulized for 2 hrs at 45°C in 0.25 ml of Nuclear Chicago Solubulizer and counted in a liquid scintillation detector.

f. Exposure to cigarette smoke

(1) Cigarettes

The cigarettes used in these studies are obtained from the University of Kentucky Tobacco and Health Institute and include the 1A1 (low nicotine) and 1R1 (high nicotine) reference cigarettes. Ten thousand cigarettes from the same lot were purchased and are stored in the original cartons plastic bags at -20°C. One week before use, the cigarettes are removed from the freezer, packages opened and stored in a humidified chamber at room temperature. Cigarettes were weighed before use so that uniformity could be maintained.

(2) Smoke exposure

Mice were exposed to cigarette smoke on the Prototype Mark II Walton Horizontal Smoke Exposure Machine. Routine calibration of the machine is made to achieve uniformity. The rotometer is set at 10. The machine is set to give a 2 sec. puff, 28 sec mix and 30 sec purge to complete the cycle. Exposure consists of the first 8 puffs of the cigarette. Twenty animals are always exposed to cigarette smoke at the same time so that equivalent exposure is given to all animals. Before each experiment the mice are acclimated to the smoking machine by putting them through 8 puff cycles, two times a day for a period of one week. Those animals to be used in smoking experiments are then exposed to increasing numbers of puffs of cigarette smoke for 2 days before they enter a smoking protocol. Sham-smoked and cage-held animals will always be used as controls.

(3) Animals

Male Balb/c mice, weighing between 13-15 grams have been used

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in the past. These animals were chosen for the following reasons. Although they contain endogenous type A and type C virus particles, spontaneous induction of leukemia is not characteristic of Balb/c mice as with other inbred strains. Secondly, these animals are low inducers of aryl hydrocarbon hydroxylase, an enzyme involved in the metabolism of aromatic hydrocarbons and thought to be related to the carcinogenesis of tobacco products. Every attempt is made to use healthy animals in these experiments. We have had some problems in the past with intestinal parasitic and worm infections in mice and have excluded these animals from the study.

3. Experimental Approach:

a. Effect of chronic exposure to cigarette smoke on humoral immunity

Groups of mice will be exposed to smoke from Kentucky Reference 1A1 and 1R1 cigarettes according to various protocols for up to 40 weeks. Following smoke exposure, the animals will be evaluated for their ability to produce serum antibody and numbers of splenic plaque-forming cells will be determined. The following parameters will be evaluated:

1) type of cigarette - 1A1 vs 1R1
2) number of cigarettes - 1,2, or 3/exposure
3) number of exposures per day - 1,2, or 3
4) duration of exposure - 10,15,20,25,30, etc., weeks
5) effect of withdrawal from smoke protocol on recovery of immune response
6) time of antigen administration
7) type of antigen used - T-dependent vs T-independent

b. Effect of chronic exposure to cigarette smoke on cell-mediated immunity

Unanue and Feldman (79) have shown that injection of macrophage bound antigen can induce a state of delayed hypersensitivity in animals. Alveolar and peritoneal macrophage will be harvested from normal animals and incubated with KLH. After washing the antigen-containing macrophages will be injected alone or in Freund's complete adjuvant into the footpads. Some 2-4 weeks later, the animals will be challenged with soluble antigen injected into the skin. The degree of erythema and induration will be measured some 24-48 hours later. These experiments will compare macrophages from smoke-treated animals and sham-smoked injected into both smoked and sham-smoked recipients.

Warr and Martin (55) have shown that alveolar macrophages from human smokers were insensitive to the action of MIF obtained from healthy volunteers and that the presence of antigen did not inhibit macrophage migration in preparations derived from antigen-sensitive smokers. Experiments will be set up to evaluate similar parameters in smoked-animals. We will determine if alveolar and peritoneal macrophages from such animals are similarly refractive to the activity of MIF generated from healthy animals. In a like manner, we will evaluate the ability of smoked animals to generate MIF to be used in assays with macrophages from normal animals. Pelley et al. (80) showed that MIF was produced in response to KLH stimulation. In order to carry out these experiments, MIF will be generated from lymph nodes of normal and smoked animals according to the procedure of Rocklin et al. (81) as used by Pelley et al. (80) lymph nodes from KLH-stimulated animals will be cultured in vitro in cell sus-

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pension. One portion of the cell suspension will be restimulated with the appropriate concentration of soluble KLH to stimulate production of MIF. Another portion of the cell suspension will be cultured in medium alone to generate control supernatant fluids. Supernatant fluids will be removed and pooled for 3 consecutive days, and cells will be resuspended in fresh tissue culture medium. The control supernatant fluids will be divided into two equal fractions. One fraction will be used unchanged while antigen will be added to a second fraction in the same concentration used for production of MIF. The supernatant pools will be centrifuged, dialyzed, lyophilized and stored at -70°C until used, at which time the sample will be reconstituted and filter sterilized before use. The MIF will be assayed with both peritoneal and alveolar macrophages obtained from smoked and control animals as described by Pelley and Schwartz (82) or by Warr and Martin (55) as adapted for alveolar macrophages.

c. Effect of vapor phase of cigarette smoke on the immune response

In view of our previous findings that 1R1 but not 1A1 cigarettes had an immunosuppressive effect in acute smoke exposure protocols we will test the effect of removing particulate materials from smoke by placing a Cambridge filter in the smoke generating machine. The first factor that must be explained is the role of nicotine in immunosuppression. If immunosuppression is still observed with vapor phase smoke of 1R1 cigarettes we can most likely rule out the effect of the high nicotine content of these cigarettes and attribute the results to differences in other organic constituents. On the other hand if immunosuppression is lost with the vapor phase then nicotine must play some possible role. Experiments will be set up to evaluate the effects of acute and chronic exposure to the vapor phase of cigarette smoke on both humoral and cell-mediated immunity as described in sections (a) and (b) above testing similar parameters.

why only nicotine as suspect?

d. Effect of cigarette smoke on the individual cell types involved in the immune response.

(1) Effect on macrophages.

(a) Phagocytic Function

Animals from which alveolar macrophages are to be obtained will be exposed to whole smoke or its vapor phase for varying periods of time. The animals will be sacrificed and macrophages will be obtained which will be tested for phagocytic function as well as the ability to participate in an immune response.

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It was observed by Cohn and Ehrenreich (83) that the pinocytosis of certain disaccharides by mouse peritoneal macrophages leads to extensive vacuolization of the macrophage cytoplasm. The vacuoles were shown to be lysosomes containing the pinocytosed disaccharide. Disaccharides which induce vacuolization were indigestible and evidently too large to escape readily from the macrophage lysosomes. Thus one has a visible marker to determine the extent of phagocytic function of a cell population.

Both macrophages obtained from smoked animals and pure cultures of alveolar and peritoneal macrophages will be prepared by the methods of adherence and density gradient centrifugation. Cultures will be exposed to smoke and/or vapor phase for varying periods of time. Initial experiments will be directed toward the determination of optimal concentration and duration of exposure to tobacco products which will allow for viable macrophage populations. The condition of the cells so treated will be assayed by morphological observation with phase contrast microscopy which will be correlated with cell viability

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as determined by the trypan blue dye exclusion test. Similar studies will be performed on alveolar macrophages obtained from animals treated with tobacco products in vivo. After exposure to tobacco products, the macrophages will be transferred to fresh media containing KLH bound to bentonite particles and following incubation for varying periods of time, the cells will be fixed in glutaraldehyde and the number of vacuoles in the cytoplasm will be enumerated and compared to untreated controls.

Once that information is obtained, treated macrophage populations will be incubated with $^{125}$I-labeled KLH and the uptake, fate and localization of this material will be evaluated as described. In these studies we will employ the various physical forms of KLH. These studies should provide information as to the effect of tobacco products on the phagocytic functions of macrophages.

(2) Immune response

It has been shown that both free macrophage-processed antigen and antigen bound to macrophage membranes after processing can be immunogenic (17) both in vivo and in vitro. The mode of presentation of antigen appears to be a function of the chemical and physical nature of the antigen.

(1) Primary immune response in vivo

In one series of experiments, macrophages will be exposed to antigen before they are treated with cigarette smoke, while in another series, the cells will be treated with the cigarette smoke before allowed to react with antigen. After various periods of incubation, the macrophages will be collected, washed to remove free antigen, and injected into nonimmune syngeneic animals. Circulating levels of antibody will be measured by the described procedures after collection of serum on various days following primary immunization. Comparison will be made with control animals which will be injected with antigen-treated macrophages which were not exposed to cigarette smoke. Similar experiments will be carried out with alveolar macrophages obtained from animals exposed to cigarette smoke in vivo.

(2) Primary immune response in vitro

As stated previously we have been working as part of the Pulmonary Program Project on an in vitro model of antibody formation patterned after the Mishell-Dutton system in which we are studying the immunologic functions of alveolar macrophages. A likely extension of this work would be to determine the effect of cigarette smoke on the ability of lymphoid organs derived from smoked-animals to participate in such a response and further to determine if macrophages from smoked-animals could restore immunological function in macrophage-depleted cultures.

(3) Secondary immune response in vivo

Animals will be injected with a primary dose of KLH in vivo. Macrophages from nonimmune animals will be exposed to cigarette smoke and antigen as described above and then injected into the primed animals. The levels of circulating antibody will be measured one week after the animals are given the secondary injection. To determine whether macrophage-associated antigen can prime animals for a secondary response, we will inject macrophages incubated with antigen either with or without treatment by cigarette smoke and then challenge with free KLH. Serum will again be collected and assayed for antibody levels.

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(4) Secondary immune response in vitro

Lymph nodes and spleens from primed animals will be removed and macrophages will be separated from the populations by standard techniques. Macrophages from smoked-exposed and control animals will be added to the lymphocyte cultures. Antibody formation will be determined by the plaque assay.

2. Effect on B and T cells

Using the reagents previous discussed (ie AMG and APCS) along with the congenitally athymic mouse system we can obtain cell populations in a relatively "pure" state to study individual cell functions. In these studies we will determine the effect of cigarette smoke on mitogenic responses. Protocols similar to those described in section 3a will be employed. The nude mouse system will also enable us to test our hypothesis that T-independent antigens are also macrophage-independent. The reasoning goes as follows: if we find that the macrophage is the prime target of the immunosuppressive effect of cigarette smoke and if our hypothesis is correct, there should be no immunosuppression observed in animals exposed to smoke and injected with a T-independent antigen. If an immunosuppressive effect is associated with the B cell, then cigarette smoke should suppress the response. By using combinations of T-dependent and T-independent antigens in nude and conventional mice we should provide further information as to the particular cells affected by cigarette smoke.

e. Effect of cigarette smoke on developmental aspects of the immune response

As stated previously, there is evidence that the immunological immaturity in the newborn of the species is not due to the lack of antibody-synthesizing cells but may primarily be due to the lack of antigen recognition or antigen processing cells in the form of competent macrophages. The studies proposed in this section will be concerned with the effects of cigarette smoke on the maturation of the immune response.

(1) Effect of cigarette smoke treatment of pregnant mice

Groups of pregnant mice will be exposed to cigarette smoke according to various protocols as described in section 3-a above. Similar groups of pregnant mice will be subjected to the stress effects of the smoking machine in the absence of cigarettes. In our basic design, pregnant mice will be exposed to smoke for one, two or three weeks of the gestation period. The time of smoke initiation will be varied, ie, the first week, the last week, the middle period etc. Litters will be assessed for immunological function at various times after birth; during early stages up to adult life. Litters will also be assessed for birth weight, litter size, and presence of deformed or defective animals. In another series of experiments litters will be divided in half, one half will receive further exposure to cigarette smoke while the others will remain untreated. Comparisons will be made with litters from sham-smoked animals. In order to determine if there are any pronounced physiological differences due to increased numbers of pregnancies, we will keep records of the number of times each female is mated and compare similar parameters of immunological function in succeeding litters from the same mother.

(2) Effect of cigarette smoke on mice of different ages

Animals will be exposed to cigarette smoke at regular intervals starting immediately after birth. Appropriate adjustments will be made for dose of cigarette smoke for neonatal animals. The animals will then be assessed for immunological function at varying time periods thereafter.

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(3) Effect of cigarette smoke on the ability of macrophages to potentiate neonatal responses

Bendinelli et al. (84) have shown that peritoneal cells were capable of potentiating the immune response of neonatal animals to SRBC. We will design similar experiments using macrophages from smoked-animals of different ages as a source of the potentiating cells. We will also use normal macrophages in smoked animals to determine if they can restore immune function.

The studies of the effects of cigarette smoke on immune function described in preceeding sections can certainly be applied to a developmental approach in that they be carried out with animals of different age.

f. Effect of cigarette smoke on lung pathology

A collaborative effect has been established with Dr. M. Robinowitz in the Department of Pathology. The following section represents his input and interests in the project.

The pathologic examination of the mice used in the above experiments will be performed to assess the presence of intercurrent infectious processes especially mycoplasma pneumonia (85). The lungs will be removed from animals involved in the various smoke exposure protocols and will be fixed by perfusion with 10% buffered formalin solution by pressure fixation (86-87). The fixed lungs will be cut sagitally so that airways can be located. Sections will be taken from the entire right and left lungs of each animal. These sections will be evaluated for the overall pulmonary architecture with special emphasis on small airway changes. The bronchi will be examined for changes in mucus glands, mucosa, and all areas will be examined for inflammatory cell numbers, distribution and pigmentation using H and E, PAS, alcian blue and trichrome stains (50,88-91). The results of the pools of controls, sham controls and smoked-animals will be tabulated and examined with a code which will not be broken until after completion of the study by one individual (M.R.). The data will be correlated with the degree and duration of smoke exposure and the status of the immune response (50).

D. Collaboration

As indicated above, a collaborative effort has been established with Dr. M. Robinowitz. This will aid us in evaluating our data in view of potential intercurrent infection of our experimental animals. Dr. Robinowitz has begun some preliminary work on lungs from our animals. He has found that from 5-10% of the animals in each of the groups (smoked, sham-smoked and bench controls) had pneumonia. Identification of these animals had removed them from our protocols. In addition, he has found, in only one case, the presence of what appeared to be a squamous cell carcinoma of the lung. At this point, the significance of this finding remains unclear. Certainly, Dr. Robinowitz' input will be an addition to this project in terms of identifying infection and may provide further insight into pathological changes in experimental animals.

Another area of collaboration that is anticipated is with Dr. P. Hamosh and his group. There are obvious areas in both of our protocols where there is common interest. Both of us are proposing studies of the effect of cigarette smoke on maturational aspects. Certainly there will be an opportunity for the sharing of common materials to be used in our immunological and their biochemical and physiological studies. Such overlaps should generate a forum for exchange of scientific thinking as well.

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F. Significance of the Proposed Research

It is well appreciated that the normal functions of the immune system protect the individual from constant challenge by foreign materials. A breakdown of immune function, whether under natural circumstances (immunological deficiency disease and aging) or induced (treatment with immunosuppressive agents) or due to environmental exposure (cigarette smoke) renders the individual subject to infection by microorganisms or prone to the development of various forms of cancer.

A potentially essential link in the chain of events leading to the initiation of both the humoral and cellular immune response is the interaction of antigen with macrophages. It is suggested that this interaction results in the processing of antigen thus rendering it immunogenic. Treatment of macrophages with various agents known to interfere with their phagocytic function results in a suppressed immune response. The proposed research should generate information which may provide insight into the possible means by which cigarette smoke may affect immune function. Since alveolar macrophages play an important role in the maintenance of lung stability, the proposed research is of direct significance to the understanding of the relationship between tobacco smoke and chronic lung disease. Further, the results of these studies should provide information which is currently unavailable concerning the effects of cigarette smoke on the maturation of the immune response.

In addition, through the proposed research, we hope to elucidate the mechanism of antigen recognition. If we can determine which physical forms of antigen are processed by macrophages, we may acquire a better understanding of why certain antigens lead to a breakdown of immune surveillance (immunological tolerance) whereas others initiate the immune response.

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# "THE ROLE OF THE MACROPHAGE IN THE IMMUNE RESPONSE: THE EFFECT OF TOBACCO PRODUCTS ON MACROPHAGE FUNCTION."

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## Abstract

Male Balb/c mice were exposed to various protocols involving acute exposure to Kentucky Reference cigarettes. The animals were exposed to smoke both before and after administration of antigen. The type, number and duration of exposures to smoke were varied. Three parameters of immune responsiveness were studied: (1) the number of direct and indirect plaque-forming cells (PFC), (2) the primary and secondary serum antibody response, and (3) the response to mitogenic stimulation.

The following findings were obtained:

1. Exposure of mice to Kentucky Reference 1A1 cigarettes in each of the various protocols tested was without effect on immune responsiveness.

2. Sham-smoked animals had a reduced immune responsiveness compared to untreated bench controls.

3. Exposure of mice to Kentucky Reference 1R1 cigarettes caused significant suppression of the appearance of both direct and indirect PFC.

4. Exposure to 2 or 3 of the 1R1 cigarettes caused greater suppression than a single cigarette.

5. Animals removed from the smoking protocol after antigen administration had a partial recovery of immune responsiveness.

6. Exposure to 1R1 cigarettes reduced the primary serum hemagglutinating antibody response, but under the conditions studied had no effect on the secondary response.

7. Exposure of mice to the 1R1 cigarettes for either 7 or 15 days had no effect on the ability of spleen cells to respond to the mitogenic stimulation of Concanavalin A or Phytohemagglutinin.

These results suggest that the smoking of high nicotine-containing cigarettes has a deleterious effect on the number of antibody-forming cells in mouse spleen populations. Further experiments are in progress to define the cells affected and to determine possible mechanisms involved.

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# Progress Report No. 3

Male [illegible] mice were exposed to various [illegible]

There have been a large number of studies carried out which have suggested that components of cigarette smoke may have direct carcinogenic activity. In addition, while containing a variety of potential carcinogens, components of cigarette smoke may also exert a suppressive effect on the immune system affecting both local immunity in the lung and systemic immunity. Therefore, the smoking of cigarettes may contribute to the initiation of a neoplastic event as well as make the individual more susceptible to infection as a consequence of chronic impairment of the normal defense mechanisms operating in the respiratory tract. In this regard, the alveolar macrophage plays a dominant role in the defense of the lung against microbial infection. It also has a prominent role in the clearance of particulate matter of many different types from the lung. Although there is local antibody (IgA) produced in the lung, the major organ of antibody formation in response to intratracheal instillation of immunogen is not the lung. It has been suggested that foreign materials deposited in the lung parenchyma are readily eliminated or rendered non-immunogenic, and that only a small proportion of the immunogen enters the blood stream and triggers an immune response in the spleen. The site of passage of the immunogenic material is thought to be in the alveolar space which is occupied by macrophages. One of the functional roles of macrophages which can be included in a long list of yet ill-defined roles, is that of transport and/or presentation of antigen to antibody-forming cells. It is very possible that the ability of the alveolar macrophage to carry out its normal defense functions might be severely affected by the presence of a wide variety of environmental pollutants that enter through the lung. Previous studies have shown that chronic exposure to sulphur dioxide and, in some cases carbon, has resulted in impairment of the immune response of experimental animals to inhaled immunogens. Another environmental pollutant, cigarette smoke, or extracts thereof, has been shown to affect the morphology of macrophages along with a number of their biochemical and functional properties. In addition, young adult cigarette smokers have been found to have significantly lower concentrations of serum antibody to influenza $A_2$ virus after either natural infection via the respiratory tract or vaccination than found in a similar group of nonsmoking controls. These studies suggested that cigarette smoking might have a detrimental effect on the immune system.

The rationale employed in these studies was based on the argument that macrophage participation in the immune response is a function of the physical nature of the immunogen. Macrophages appear to be necessary for immune responses to multideterminant, complex immunogens. In addition, immune responses to these materials also require the cooperative effect of thymus-derived cells. Therefore, the use of a complex antigen such as SRBC or KLH, and the observation of a suppressed immune response following exposure to smoke, would enable us to study mechanisms, at the cellular level.

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The initial period of this grant was spent in establishing a system in which we could measure antibody production by single cells against KLH. Details of the hemolytic plaque assay used for the detection and enumeration of individual cells either synthesizing or releasing antibody to KLH were presented in a previous

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progress report and the published paper is appended to the grant renewal request. In the second year of the grant, we attempted to establish techniques in which heterogeneous cell populations could be separated into individual functional cell types. Our particular interest at that time was directed toward the preparation of a potent and specific anti-macrophage serum to be used to deplete cell populations of macrophages so that we could determine whether the immune response to KLH was dependent upon the presence of accessory cells. Details of our work with rabbit anti-mouse macrophage serum were given in Progress Report No. 2 and a copy of our manuscript which was recently submitted to Immunology is appended. We extended the anti-macrophage serum work to the rabbit system and have established that the in vitro anamnestic response of rabbit popliteal lymph node cells is also macrophage-dependent. A copy of an abstract presented at the 59th Annual Meeting of the American Association of Immunologists in April, 1975 is also included in the grant application. Also, during the second year of the grant we extended out studies of the fundamental concepts of antibody formation into areas peripheral to the mission of this grant but which may have some bearing on our results. In Progress Report No. 2 we provided some information on the use of an anti-plasma cell serum to study cellular differentiation of the murine plasma cell. A copy of the first of at least three publications from this work, submitted (Journal of Immunology) or to be submitted, is attached. These studies have also led us into work with T-independent antigens and served as impetus for us to begin a colony of nude (Thymus-deficient, nu/nu) mice. In a second series of studies, we were interested in studying potential regulatory mechanisms operating in the immune response. We have observed that Prostaglandin $E_1$, but not other prostaglandins, enhanced antibody formation in our in vitro anamnestic response system. These studies could eventually be extended to immunocompetent cells found in lung tissue. A copy of our manuscript submitted to Prostaglandins is attached to the grant application.

The experiments described in this Progress Report were initiated in late October, 1974 upon receipt of the Prototype Mark II Walton Horizontal Smoke Exposure Machine. Initial attempts were made to calibrate the machine to [illegible] settings indicated in the manual. Rotometer was set at 14 to achieve a 35 ml puff volume, purge set at 10. The machine was set to give a 2 sec. puff, 28 sec. mix and 30 sec. purge to complete the cycle. All experiments were done with Kentucky Reference cigarettes, either 1A1 or 1R1, which had been stored at -20°C prior to use. One week before use, the cigarettes were removed from the freezer, packages opened and stored in a humidified chamber at room temperature. Exposure consisted of the first 8 puffs from the cigarettes. Male Balb/c mice were used as the experimental animal for several reasons. Although they contain endogeneous type A and type C virus particles, spontaneous induction of leukemia is not characteristic of Balb/c mice as with other inbred strains. Secondly, Dr. Kreisher suggested that male Balb/c mice be used due to low inducibility of aryl hydrocarbon hydroxylase, an enzyme involved in the metabolism of aromatic hydrocarbons and thought to be related to the carcinogenesis of tobacco products. Before each experiment, the animals were acclimated to the smoking machine by putting them through 8 puff cycles, two times a day for a period of one week. Those animals that were to be used in smoking experiments were then exposed to increasing numbers of puffs of cigarette smoke for 2 days before they entered a smoking protocol. It should be noted that there were few deaths, probably due to intoxication when the 1A1 cigarettes were used. A mortality



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rate of about 15-20% was obtained using 1R1 cigarettes. As will become evident as the data is discussed, the sham-smoked animals responded in a diminished fashion when compared to the untreated, bench control animals. All other methods are as described in the grant application. Our initial experiments were concerned with obtaining an understanding of the effects of cigarette smoke on the number of spleen plaque-forming cells which is a direct measure of antibody-forming cells, on serum antibody levels in both primary and secondary immune responses and on the ability of spleen cells to respond to mitogenic stimulation. These experiments have concentrated on acute phase of smoke exposure with antigen being injected before, along with or after exposure to smoke. Some of the parameters studied were type of cigarette (1A1 vs. 1R1), the number of cigarettes used, duration of exposure to smoke and effect of withdrawal of animals from smoking protocols.

In the first series of experiments, male Balb/c mice were injected with KLH on day 0 and smoking of one 1A1 cigarette twice each day for 5 days was begun at the same time. On the 5th day, spleens were removed from each of 6 smoked, 6 sham-smoked and 4 bench control animals. Single cell suspensions were prepared and spleens were assayed individually for direct (IgM) and indirect (IgG) PFC. Table 1 shows that both smoked and sham-smoked animals had a loss of weight after being placed on the smoking machine. Again, it will be pointed out that there was a reduction in the number of PFC even in sham-smoked animals compared to bench controls. These results indicate that some of the suppression of immune responsiveness may be due to the stress conditions placed on experimental animals. The number of PFC in smoked and sham-smoked animals is not significantly different indicating that the approximate 25% suppression of responsiveness is probably due to stress conditions. Tables 2 and 3 show the results of experiments in which animals were exposed to smoke as above for 7 days and then injected with KLH. In the former experiment the animals were rested (no smoke) for the following 5 days, while, in the latter, smoke was continued according to the same protocol until the time of assay, day 12. It should be noted that in neither was there significant suppression of the immune response in smoked animals.

In the next series of experiments, animals were injected with KLH on day 0 and smoke exposure was begun 1, 2, 3 or 4 days later and continued until the time of assay, day 5. It can be seen in Tables 4-7 that there was no immunosuppressive effect of smoking a single 1A1 cigarette twice each day when direct PFC were enumerated. In some cases, Tables 5 and 7, it appears that smoking suppressed antibody formation; however, sham-smoked animals also had depressed immune responses, again suggesting that the reduction in immunocompetence was a function of stress conditions.

In view of the findings that a single 1A1 cigarette was ineffective in having an influence on the immune response of the mice we decided to increase the number of cigarettes used for each exposure. One possible reason for lack of effect of the 1A1 could have been related to dose of smoke that the animals received. Table 8 shows the effect of giving antigen 1 day before exposure to 2 cigarettes. It can be seen that increasing the smoke dose of 1A1 cigarettes



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had no effect on the immune responsiveness of test animals. Table 9 shows the results of experiments in which mice were exposed to smoke for 5 days, then injected with KLH and were rested for the time before spleens were assayed for PFC. It can be seen that increasing the smoke dose had no effect on either direct or indirect PFC. Although not shown, a similar experimental design in which the animals were exposed to smoke from 2 cigarettes both before and after antigen administration resulted in a similar lack of effect on the PFC capabilities of the experimental animals. The dose of smoke was then increased by exposing the animals to three 1A1 cigarettes twice each day. Table 10 shows the results of experiments in which the animals were injected with KLH at time 0 and smoke was given at the same time and for the following 5 days. It can be seen that such an increase in smoke exposure did not have a significant effect on the number of PFC since the sham-smoked animals also showed a decrease in immune responsiveness compared to control, untreated mice. Table 11 shows an experiment in which mice were exposed to three 1A1 cigarettes before the administration of antigen and then rested until the time of assay, day 10. Again, it can be seen that the apparent diminution of immune responsiveness is not significant since the sham-smoked animals also showed a decreased immune response. Not shown are the results of a similar experiment in which mice were also exposed to the three 1A1 cigarettes after antigen injection. In this case as well there was no clear cut effect of smoking on the ability of the mice to respond to KLH. Table 12 presents data from an experiment in which mice were exposed to smoke from three 1A1 cigarettes and antigen was injected one day later. This experiment provides some indication (which must be interpreted cautiously) that the three 1A1 cigarettes had an immunosuppressive effect on both direct and indirect PFC. It should be pointed out that these animals were larger than any used previously, and, therefore, could have tolerated the stress effects of the smoking machine better than smaller animals. The immune response of smoked animals was suppressed by about 30% whereas sham-smoked animals showed only 10% suppression compared to the untreated controls. Several possible factors could explain the reproducible lack of effect of the 1A1 cigarettes on the immune response. First, the nature of the components of the 1A1 cigarette may preclude them being immunosuppressive, since these cigarettes are low nicotine-containing. Second, the duration of exposure to smoke may have been too short to see a systemic suppressive immunosuppressive effect. Third, cigarette smoke may not be immunosuppressive.

We next turned our attention to the effects of 1R1 cigarettes on PFC production since these cigarettes are high nicotine-containing. Table 13 shows the results of an experiment in which mice were exposed to a single 1R1 cigarette starting at the time of antigen administration. All of the conditions used for smoke exposure were the same as those described for the 1A1 cigarettes. It can be seen that there was a significant suppression of the direct plaque-forming cell number in smoked animals. Smoked animals showed a 76% suppression of PFC compared to untreated bench controls. In this experiment, as in preceeding ones, there was also about a 10-15% suppression in the sham-smoked animals. However, there is certainly a significant reduction of PFC in smoked animals compared to these controls. Table 14 shows that if the animals were exposed to smoke before antigen injection and then allowed to rest (i.e., no addition smoke, but being



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placed on the smoking machine) until the day of assay there appears to be restoration of immune responsiveness. It can be seen that there is only 40% suppression of responsiveness if the animals are not exposed to smoke for the 4-5 days after antigen administration. These results suggest that the effects of smoking may be reversible. Table 15 shows the effect of increasing smoke exposure (2 1R1 cigarettes) in immune responsiveness. In this experiment the animals were given antigen and started on the smoking protocol at the same time. The data indicate that exposure to smoke for 5 days resulted in a 98% suppression of direct PFC and complete suppression of the appearance of indirect PFC. Again I must point out that there was an effect of stress on control animals. Tables 16 and 17 show the effects of exposing the animals to smoke before antigen injection and then withholding or continuing the smoking protocol until the day of assay. Table 16 shows that there is an approximate 50% recovery of the PFC number in animals which were no longer exposed to smoke. Table 17, however, shows that continued exposure to smoke after antigen injection results in almost complete suppression of the appearance of plaque-forming cells. Table 18 shows the effect of increasing the number of 1R1 cigarettes to 3 at each exposure. In this experiment the mice were injected with KLH and started on the smoking protocol at the same time. Again there was almost complete suppression of both direct and indirect PFC in the smoked animals. Tables 19 and 20 give the results of experiments in which the animals were exposed to smoke from three 1R1 cigarettes for 5 days preceeding antigen injection and were either exposed to smoke for an additional 5 days or withdrawn from the smoking protocol. If smoke was continued (Table 20), then complete suppression of PFC appearance was obtained. On the other hand, if smoked was withheld, there appeared to be recovery of immune responsiveness (Table 19). In view of these findings, we decided that for the near future all additional experiments on the effects of cigarette smoke would employ 1R1 cigarettes.

suppression compared to the untreated controls. Several possible factors could

Preliminary experiments were carried out on the effect of smoke exposure on serum hemagglutinating antibody. Animals were exposed to three 1R1 cigarettes for varying periods up to 28 days. At intervals of 7 days, groups of 10 mice each were injected with KLH and were then bled 7 days later. Table 21 shows that there was significant diminution of serum antibody against KLH in animals even after one week of smoke exposure. Continued exposure resulted in continued suppression of antibody formation. The results in Table 22 indicate that there is recovery of the serum antibody response in animals which had been removed from the smoking protocol. These effects are similar to that observed in the PFC response to 1R1 cigarettes. One experiment was also performed on the effect of smoking on the secondary response. In this case, animals were injected on day 0 and then again on day 28 without ever having been exposed to smoke. Groups of 10 mice each were then exposed to three 1R1 cigarettes for either 4, 6 or 8 days following secondary antigen stimulation and their serum antibody levels were determined. The data in Table 23 indicate that there was no immunosuppressive effect on the in vivo secondary response. This finding may possibly be explained by the fact that the population of memory cells generated as a consequence of primary immunization is resistant to the immunosuppressive effect of cigarette smoke. Additional experiments in which animals are smoked before secondary immunization are now in progress.

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Preliminary experiments were also undertaken to determine if there was a similar suppressive effect of cigarette smoke on T-cell functions. In these experiments mice were exposed to cigarette smoke from three 1R1 cigarettes, two times each day for either 7 or 15 days consecutively. At the end of this period, the mice were sacrificed, their spleens removed, cell suspension prepared and set up in culture. Cultures were stimulated with optimal dilutions of either Concanavalin A (2.0 μg) or Phytohemagglutinin (25 μg) and incubated for 48 hrs. After this period, 1 μCi of $^3$H-thymidine was added to mitogen-stimulated and unstimulated control cultures and incubation was continued for an additional 24 hrs. After this, cultures were harvested, the cells were washed, precipitated with TCA and prepared for liquid scintillation counting. The data in Tables 24 and 25 indicate that there was no suppression of T-cell function as a consequence of exposure to cigarette smoke. On the contrary, the data in Table 24 suggests that there is an enhancement of mitogenic activity in smoke exposed animals. Whether this enhancement is real will await reproduction of the experiment. The lack of a suppressive effect on T-cell function was not unexpected in such short term smoke exposure protocols since similar observations had been reported previously.

Finally, I should like to point out three areas of research which are now in progress and have not been included in this report. We have been exposing several groups of mice to both 1A1 and 1R1 cigarettes on a routine basis of 5 days/week, 2 exposures/day since October, 1974 when the machine first arrived. At some time in the near future, these animals will be subjected to similar assays described to determine the effects of chronic smoke exposure. Secondly, we are exposing a group of mice to the 1R1 cigarettes in the presence of a Cambridge filter to evaluate the effect of the gas phase of smoke. Lastly, we have established a collaborative effort with Dr. M. Robinowitz, a member of the Department of Pathology, who is interested in evaluating pathological changes in animals exposed to smoke. His many interests are concerned with detecting inflammatory changes and detecting pigment in macrophages found in terminal bronchioles. For these studies, we are giving him lungs from mice used in our experimental protocols.

We feel that we now have the beginnings to undertake comprehensive studies of mechanisms of defects induced by cigarette smoke. The observation that 1R1 cigarettes caused suppression of immune responsiveness in the intact animal leads us to ask where or upon which cell type the suppressive effect is operating. Once determined, we can shift our emphasis to the molecular level.

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## Table 1

**Effect of Acute Exposure to Cigarette Smoke on the In Vivo Primary PFC Response**

**Antigen Given at Time of Initiation of Smoking**

| Group (n) | Body Weight at Start | Body Weight at Assay | Spleen Weight at Assay | Direct PFC/$10^6$ cells | Indirect PFC/$10^6$ cells |
|---|---|---|---|---|---|
| Smoked (6) | 15.26 | 13.13 | 0.075 | 1,603 | N.D. |
| Sham-smoked (6) | 13.36 | 14.95 | 0.094 | 1,750 | N.D. |
| Bench Control (4) | 14.28 | 15.31 | 0.104 | 2,016 | N.D. |

N.D. = Not Determined

**Conditions:**

**Cigarette:** Kentucky Reference 1A1

**Exposure:** 1 Cigarette, 2 times/day, 0-5 days

**Antigen:** Injected on day 0

**Assay:** Day 5

**Additional Information:** Antigen given day 0, smoke begun day 0

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Table 2

Effect of Acute Exposure to Cigarette Smoke
on the In Vivo Primary PFC Response

Smoke Before Antigen
No Smoke After Antigen

| Group (n) | Body Weight at Start | Body Weight at Assay | Spleen Weight at Assay | Direct PFC/$10^6$ cells | Indirect PFC/$10^6$ cells |
|---|---|---|---|---|---|
| Smoked (5) | 14.38 | 14.58 | 0.104 | 2,022 | 677 |
| Sham-smoked (5) | 14.60 | 13.43 | 0.102 | 2,250 | 424 |
| Bench Control (3) | 14.53 | 14.06 | 0.108 | 2,246 | 512 |

Conditions:

Cigarette: Kentucky Reference 1A1

Exposure: 1 Cigarette, 2 times/day, 0-7 days

Antigen: Injected on day 7

Assay: Day 12

Additional Information: Animals rested (no smoke) days 7-12

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Source: https://www.industrydocuments.ucsf.edu/docs/xnnm0000

Table 3

Effect of Acute Exposure to Cigarette Smoke
on the In Vivo Primary PFC Response

Smoke Before Antigen
Smoke After Antigen

| Group (n) | Body Weight at Start | Body Weight at Assay | Spleen Weight at Assay | Direct PFC/$10^6$ cells | Indirect PFC/$10^6$ cells |
|---|---|---|---|---|---|
| Smoked (5) | 14.00 | 14.32 | 0.100 | 1,780 | 585 |
| Sham-smoked (5) | 14.60 | 13.43 | 0.102 | 1,921 | 521 |
| Bench Control (3) | 14.52 | 14.96 | 0.106 | 2,142 | 553 |

Conditions:

Cigarette: Kentucky Reference 1A1

Exposure: 1 Cigarette, 2 times/day, 0-7 days

Antigen: Injected on day 7

Assay: Day 12

Additional Information: Animals smoked on days 7-12

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Table 4

Effect of Acute Exposure to Cigarette Smoke
on the In Vivo Primary PFC Response

Antigen Injected Day 0
Smoke Begun 1 Day Later

| Group (n) | Body Weight at Start | Body Weight at Assay | Spleen Weight at Assay | Direct PFC/$10^6$ cells | Indirect PFC/$10^6$ cells |
|---|---|---|---|---|---|
| Smoked (6) | 15.93 | 14.95 | 0.098 | 1,716 | N.D. |
| Sham-smoked (4) | 15.03 | 15.80 | 0.106 | 1,640 | N.D. |
| Bench Control (3) | 15.61 | 15.85 | 0.112 | 1,987 | N.D. |

N.D. = Not Determined

Conditions:

Cigarette: Kentucky Reference 1A1

Exposure: 1 Cigarette, 2 times/day, days 1-5

Antigen: Injected on day 0

Assay: Day 5

Additional Information: Antigen given day 0, smoke begun 1 day later

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Table 5

Effect of Acute Exposure to Cigarette Smoke
on the In Vivo Primary PFC Response

Antigen Injected Day 0
Smoke Begun 2 Days Later

| Group (n) | Body Weight at Start | Body Weight at Assay | Spleen Weight at Assay | Direct PFC/$10^6$ cells | Indirect PFC/$10^6$ cells |
|---|---|---|---|---|---|
| Smoked (5) | 17.10 | 15.84 | 0.113 | 1,653 | N.D. |
| Sham-smoked (5) | 16.90 | 16.47 | 0.126 | 1,735 | N.D. |
| Bench Control (3) | 17.23 | 17.98 | 0.124 | 2,347 | N.D. |

N.D. = Not Determined

Conditions:

Cigarette: Kentucky Reference 1A1

Exposure: 1 Cigarette, 2 times/day, days 2-5

Antigen: Injected day 0

Assay: Day 5

Additional Information: Antigen given day 0, smoke begun 2 days later

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Table 6

Effect of Acute Exposure to Cigarette Smoke
on the In Vivo Primary PFC Response

Antigen Injected Day 0
Smoke Begun 3 Days Later

| Group (n) | Body Weight at Start | Body Weight at Assay | Spleen Weight at Assay | Direct PFC/$10^6$ cells | Indirect PFC/$10^6$ cells |
|---|---|---|---|---|---|
| Smoked (5) | 17.58 | 16.34 | 0.116 | 1,986 | N.D. |
| Sham-smoked (6) | 16.32 | 15.76 | 0.122 | 2,078 | N.D. |
| Bench Control (4) | 17.23 | 16.94 | 0.112 | 2,272 | N.D. |

N.D. = Not Determined

Conditions:

Cigarette: Kentucky Reference 1A1

Exposure: 1 Cigarette, 2 times/day, days 3-5

Antigen: Injected day 0

Assay: Day 5

Additional Information: Antigen given day 0, smoke begun 3 days later

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Table 7

Effect of Acute Exposure to Cigarette Smoke
on the In Vivo Primary PFC Response

Antigen Injected Day 0
Smoke Begun 4 Days Later

| Group (n) | Body Weight at Start | Body Weight at Assay | Spleen Weight at Assay | Direct PFC/$10^6$ cells | Indirect PFC/$10^6$ cells |
|---|---|---|---|---|---|
| Smoked (5) | 15.48 | 15.86 | 0.112 | 1,516 | N.D. |
| Sham-smoked (5) | 18.16 | 16.66 | 0.117 | 1,677 | N.D. |
| Bench Control (4) | 16.73 | 17.21 | 0.123 | 2,034 | N.D. |

N.D. = Not Determined

Conditions:

Cigarette: Kentucky Reference 1A1

Exposure: 1 Cigarette, 2 times/day, days 4-5

Antigen: Injected on day 0

Assay: Day 5

Additional Information: Antigen given day 0, smoke begun 4 days later

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Table 8

Effect of Acute Exposure to Cigarette Smoke
on the In Vivo Primary PFC Response

Antigen Given 1 Day Before Smoke Begun
(2 Cigarettes)

| Group (n) | Body Weight at Start | Body Weight at Assay | Spleen Weight at Assay | Direct PFC/$10^6$ cells | Indirect PFC/$10^6$ cells |
|---|---|---|---|---|---|
| Smoked (5) | 14.92 | 12.82 | 0.083 | 2,101 | 521 |
| Sham-smoked (5) | 14.96 | 14.20 | 0.101 | 2,069 | 586 |
| Bench Control (4) | 14.85 | 15.13 | 0.106 | 2,393 | 647 |

Conditions:

Cigarette: Kentucky Reference 1A1

Exposure: 2 Cigarettes, 2 times/day, 0-5 days

Antigen: Injected on day 0

Assay: Day 5

Additional Information: Antigen given 1 day before smoke begun

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Source: https://www.industrydocuments.ucsf.edu/docs/xndm0060

## Table 9

### Effect of Acute Exposure to Cigarette Smoke on the In Vivo Primary PFC Response

Smoke Before Antigen (2 Cigarettes)
No Smoke After Antigen

| Group (n) | Body Weight at Start | Body Weight at Assay | Spleen Weight at Assay | Direct PFC/$10^6$ cells | Indirect PFC/$10^6$ cells |
|---|---|---|---|---|---|
| Smoked (5) | 15.02 | 12.28 | 0.082 | 1,966 | 406 |
| Sham-smoked (4) | 15.00 | 14.20 | 0.078 | 1,872 | 421 |
| Bench Control (4) | 15.61 | 16.02 | 0.102 | 2,153 | 648 |

Conditions:

| | |
|---|---|
| Cigarette: | Kentucky Reference 1A1 |
| Exposure: | 2 Cigarettes, 2 times/day, 0-5 days |
| Antigen: | Injected on day 6 |
| Antigen: | Injected on day 0 |
| Assay: | Day 11 |
| Additional Information: | Animals rested (no smoke) days 6-11 |

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Source: https://www.industrydocuments.ucsf.edu/docs/ynbm0078

Table 9

Table 10

Effect of Acute Exposure to Cigarette Smoke
on the In Vivo Primary PFC Response

Smoke Before Antigen (3 Cigarettes)
Antigen and Smoke Begun on Day 0
(3 Cigarettes)

| Group (n) | Body Weight at Start | Body Weight at Assay | Spleen Weight at Assay | Direct PFC/$10^6$ cells | Indirect PFC/$10^6$ cells |
|---|---|---|---|---|---|
| Smoked (5) | 12.44 | 12.24 | 0.075 | 1,575 | N.D. |
| Sham-smoked (5) | 12.86 | 13.40 | 0.091 | 1,627 | N.D. |
| Bench Control (5) | 12.68 | 13.01 | 0.086 | 1,964 | N.D. |

N.D. = Not Determined

Conditions:

Cigarette: Kentucky Reference 1A1

Exposure: 3 Cigarettes, 2 times/day, days 0-5

Antigen: Injected on day 0

Assay: Day 5

Additional Information: Antigen and smoke begun on day 0

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Source: https://www.industrydocuments.ucsf.edu/docs/xndm0000

Table 11

Effect of Acute Exposure to Cigarette Smoke on the In Vivo Primary PFC Response

Smoke Before Antigen (3 Cigarettes)
No Smoke After Antigen

| Group (n) | Body Weight at Start | Body Weight at Assay | Spleen Weight at Assay | Direct PFC/$10^6$ cells | Indirect PFC/$10^6$ cells |
|---|---|---|---|---|---|
| Smoked (5) | 12.40 | 12.74 | 0.092 | 1,531 | N.D. |
| Sham-smoked (5) | 12.40 | 13.53 | 0.100 | 1,742 | N.D. |
| Bench Control (5) | 12.81 | 13.92 | 0.106 | 2,165 | N.D. |

N.D. = Not Determined

Conditions:

Cigarette: Kentucky Reference 1A1

Exposure: 3 Cigarettes, 2 times/day, day 0-5

Antigen: Injected day 5

Assay: Day 10

Additional Information: Animals rested (no smoke) days 5-10

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Source: https://www.industrydocuments.ucsf.edu/docs/xndm0000

Table 12

Effect of Acute Exposure to Cigarette Smoke
on the In Vivo Primary PFC Response

Antigen Injected 1 Day After Smoke Begun
(3 Cigarettes)

| Group (n) | Body Weight at Start | Body Weight at Assay | Spleen Weight at Assay | Direct PFC/$10^6$ cells | Indirect PFC/$10^6$ cells |
|---|---|---|---|---|---|
| Smoked (5) | 17.92 | 16.82 | 0.112 | 1,467 | 424 |
| Sham-smoked (5) | 18.93 | 17.96 | 0.111 | 1,890 | 591 |
| Bench Control (3) | 18.21 | 19.03 | 0.121 | 2,093 | 638 |

Conditions:

Cigarette: Kentucky Reference 1A1

Exposure: 3 Cigarettes, 2 times/day, days 0-5

Antigen: Injected day 1

Assay: Day 5

Additional Information: Antigen injected 1 day after smoke begun

100345816

Source: https://www.industrydocuments.ucsf.edu/docs/xxxxxxxx

Table 13

Effect of Acute Exposure to Cigarette Smoke on the In Vivo Primary PFC Response

Antigen and Smoke Given at Same Time (1 Cigarette)

| Group (n) | Body Weight at Start | Body Weight at Assay | Spleen Weight at Assay | Direct PFC/$10^6$ cells | Indirect PFC/$10^6$ cells |
|---|---|---|---|---|---|
| Smoked (5) | 15.32 | 13.36 | 0.089 | 486 | N.D. |
| Sham-smoked (5) | 15.06 | 12.86 | 0.079 | 1,827 | N.D. |
| Bench Control (5) | 15.16 | 16.10 | 0.108 | 2,084 | N.D. |

N.D. = Not Determined

Conditions:

Cigarette: Kentucky Reference 1R1

Exposure: 1 Cigarette, 2 times/day, days 0-5

Antigen: Injected day 0

Assay: Day 5

Additional Information: None

100354817

Source: https://www.industrydocuments.ucsf.edu/docs/...

Table 14

Effect of Acute Exposure to Cigarette Smoke
On the In Vivo Primary PFC Response

Smoked Before Antigen (1 Cigarette)
No Smoke After Antigen

| Group (n) | Body Weight at Start | Body Weight at Assay | Spleen Weight at Assay | Direct PFC/$10^6$ cells | Indirect PFC/$10^6$ cells |
|---|---|---|---|---|---|
| Smoked (5) | 15.26 | 14.42 | 0.084 | 1,143 | N.D. |
| Sham-smoked (5) | 15.20 | 12.68 | 0.062 | 1,621 | N.D. |
| Beach Control (5) | 15.24 | 16.38 | 0.101 | 1,857 | N.D. |

N.D. = Not Determined

Conditions:

Cigarette: Kentucky Reference 1R1

Exposure: 1 Cigarette, 2 times/day, days 0-3

Antigen: Injected on day 5

Assay: Day 10

Additional Information: Animals rested (no smoke) days 5-10

100354818

Source: https://www.industrydocuments.ucsf.edu/docs/xndm0000

Table 15

Effect of Acute Exposure to Cigarette Smoke
on the In Vivo Primary PFC Response

Antigen and Smoke Given at Same Time
(2 Cigarettes)

| Group (n) | Body Weight at Start | Body Weight at Assay | Spleen Weight at Assay | Direct PFC/$10^6$ cells | Indirect PFC/$10^6$ cells |
|---|---|---|---|---|---|
| Smoked (5) | 15.40 | 14.06 | 0.084 | 42 | 0 |
| Sham-smoked (5) | 15.40 | 13.80 | 0.108 | 1,415 | 486 |
| Bench Control (5) | 15.42 | 16.82 | 0.115 | 1,986 | 621 |

Conditions:

Cigarette: Kentucky Reference 1R1

Exposure: 2 Cigarettes, 2 times/day, days 0-5

Antigen: Injected day 0

Assay: Day 5

Additional Information: Antigen and smoke given same time

100354819

Source: https://www.industrydocuments.ucsf.edu/docs/xndm0000

Table 16

Effect of Acute Exposure to Cigarette Smoke
on the In Vivo Primary PFC Response

Animals Smoked Before Antigen (2 Cigarettes)
Animals Rested (No Smoke) After Antigen

| Group (n) | Body Weight at Start | Body Weight at Assay | Spleen Weight at Assay | Direct PFC/$10^6$ cells | Indirect PFC/$10^6$ cells |
|---|---|---|---|---|---|
| Smoked (6) | 15.34 | 14.16 | 0.113 | 1,063 | 286 |
| Sham-smoked (4) | 15.25 | 13.65 | 0.110 | 1,996 | 451 |
| Bench Control (4) | 15.28 | 16.82 | 0.121 | 2,154 | 683 |

Conditions:

Cigarette: Kentucky Reference 1R1

Exposure: 2 Cigarettes, 2 times/day, days 0-5

Antigen: Injected on day 5

Assay: Day 10

Additional Information: Animals rested (no smoke) days 5-10

100345820

Source: https://www.industrydocuments.ucsf.edu/docs/xndm0000

Table 17

Effect of Acute Exposure to Cigarette Smoke on the In Vivo Primary PFC Response

Animals Smoked Before Antigen (2 Cigarettes)
Animals Smoked After Antigen (2 Cigarettes)

| Group (n) | Body Weight at Start | Body Weight at Assay | Spleen Weight at Assay | Direct PFC/$10^6$ cells | Indirect PFC/$10^6$ cells |
|---|---|---|---|---|---|
| Smoked (6) | 12.62 | 11.43 | 0.071 | 1.55 | 0 |
| Sham-smoked (8) | 12.72 | 11.90 | 0.088 | 1,743 | 387 |
| Bench Control (3) | 12.68 | 13.72 | 0.124 | 2,087 | 592 |

Conditions:

Cigarette: Kentucky Reference 1R1

Exposure: 2 Cigarettes, 2 times/day, days 0-5

Antigen: Injected on day 5

Assay: Day 10

Additional Information: Smoke continued until day 10

100345821

Source: https://www.industrydocuments.ucsf.edu/docs/yndm0000

Table 18

## Effect of Acute Exposure to Cigarette Smoke on the In Vivo Primary PFC Response

Animals Smoked Before Antigen (2 Cigarettes)
Antigen and Smoke Given at Day 0
(3 Cigarettes)

| Group (n) | Body Weight at Start | Body Weight at Assay | Spleen Weight at Assay | Direct PFC/$10^6$ cells | Indirect PFC/$10^6$ cells |
|---|---|---|---|---|---|
| Smoked (6) | 16.06 | 15.22 | 0.126 | 21 | 0 |
| Sham-smoked (4) | 16.20 | 15.76 | 0.153 | 2,671 | 821 |
| Bench Control (5) | 16.23 | 17.20 | 0.162 | 2,866 | 924 |

**Conditions:**

**Cigarette:** Kentucky Reference 1R1

**Exposure:** 3 Cigarettes, 2 times/day, days 0-5

**Antigen:** Injected day 0

**Assay:** Day 5

**Additional Information:** None

100354822

Source: https://www.industrydocuments.ucsf.edu/docs/xndm0000

Table 19

Effect of Acute Exposure to Cigarette Smoke on the In Vivo Primary PFC Response

Smoke Before Antigen (3 Cigarettes)
No Smoke After Antigen

| Group (n) | Body Weight at Start | Body Weight at Assay | Spleen Weight at Assay | Direct PFC/$10^6$ cells | Indirect PFC/$10^6$ cells |
|---|---|---|---|---|---|
| Smoked (6) | 14.28 | 13.81 | 0.082 | 1,468 | N.D. |
| Sham-smoked (4) | 14.31 | 13.90 | 0.084 | 2,320 | N.D. |
| Bench Control (4) | 14.24 | 15.16 | 0.099 | 2,613 | N.D. |

N.D. = Not Determined

Conditions:

| | |
|---|---|
| Cigarette: | Kentucky Reference 1R1 |
| Exposure: | 3 Cigarettes, 2 times/day, days 0-5 |
| Antigen: | Injected on day 5 |
| Assay: | Day 10 |
| Additional Information: | Animals rested (no smoke) days 5-10 |

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Source: https://www.industrydocuments.ucsf.edu/docs/xndm0000

Table 20

Effect of Acute Exposure to Cigarette Smoke
on the In Vivo Primary PFC Response

Smoked Before Antigen (3 Cigarettes)
Smoked After Antigen (3 Cigarettes)

| Group (n) | Body Weight at Start | Body Weight at Assay | Spleen Weight at Assay | Direct PFC/$10^6$ cells | Indirect PFC/$10^6$ cells |
|---|---|---|---|---|---|
| Smoked (5) | 15.16 | 14.21 | 0.099 | 26 | N.D. |
| Sham-smoked (5) | 15.14 | 14.38 | 0.110 | 2,216 | N.D. |
| Bench Control (5) | 15.32 | 16.07 | 0.123 | 2,408 | N.D. |

N.D. = Not Determined

Conditions:

| | |
|---|---|
| Cigarette: | Kentucky Reference 1R1 |
| Exposure: | 3 Cigarettes, 2 times/day, days 0-5 |
| Antigen: | Injected on day 5 |
| Assay: | Day 10 |
| Additional Information: | Animals smoked days 5-10 |

100345824

Source: https://www.industrydocuments.ucsf.edu/docs/xndm0080

Table 21

Effect of Acute Exposure to Cigarette Smoke
on the In Vivo Primary Serum Antibody Response

Smoked Before Antigen (3 Cigarettes)
Smoked After Antigen (3 Cigarettes)

| Day Antigen Injected | Day of Assay | HA Titer ($Log_2$) | | |
|---|---|---|---|---|
| | | Smoked | Sham-smoked | Bench Control |
| 0 | 7 | 4 | 8 | 9 |
| 7 | 14 | 4 | 9 | 10 |
| 14 | 21 | 4 | 8 | 9 |
| 21 | 28 | 3 | 8 | 9 |
| 28 | 35 | 3 | 9 | 10 |

Conditions:
10 mice/group
Cigarette: Kentucky Reference 1R1
Exposure: 3 Cigarettes, 2 times/day, day 0-35

Additional Information: Animals exposed to smoke throughout entire period.

100345825

Source: https://www.industrydocuments.ucsf.edu/docs/xndm0000

Table 22

Effect of Acute Exposure to Cigarette Smoke on the In Vivo Primary Serum Antibody Response

Effect of Acute Exposure to Cigarette Smoke on the In Vivo Primary Serum Antibody Response

| Day Antigen Injected | Day of Assay | Smoked | HA Titer ($Log_2$) Sham-smoked | Bench Control |
|---|---|---|---|---|
| 0 | 7 | 4 | 8 | 9 |
| 7 | 14 | 6 | 9 | 10 |
| 14 | 21 | 8 | 10 | 10 |
| 21 | 28 | 9 | 9 | 10 |
| 28 | 35 | 9 | 9 | 10 |

**Conditions:** 10 mice/group

**Cigarette:** Kentucky Reference 1R1

**Exposure:** 3 Cigarettes, 2 times/day, days 0-7

**Additional Information:** Animals not smoked from days 7-35

1003545826

Source: https://www.industrydocuments.ucsf.edu/docs/xndm0000

Table 23

Effect of Acute Exposure to Cigarette Smoke
on the In Vivo Secondary Serum Antibody Response

| Days of Smoke Exposure | HA Titer ($Log_2$) | | |
|---|---|---|---|
| | Smoked | Sham-smoked | Bench Control |
| 28-35 | 11 | 11 | 12 |
| 30-35 | 11 | 11 | 12 |
| 32-35 | 11 | 11 | 12 |

Conditions: 10 mice/group
All mice injected day 0
All mice reinjected day 28
Assay done on day 35

Cigarette: Kentucky Reference 1R1

Exposure: 3 Cigarettes, 2 times/day

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Source: https://www.industrydocuments.ucsf.edu/docs/xndm0000

Table 24

## Effect of Acute Exposure (7 Days) to Smoke on the Response to Mitogenic Stimulation

| Group | $^3$H-Thymidine Incorporated (cpm) Concanavalin A | | Phytohemagglutinin | |
|---|---|---|---|---|
| Unstimulated | 1,843 | | 1,843 | |
| Smoked | 35,520 | (19.3) | 23,626 | (12.8) |
| Unstimulated | 1,935 | | 1,935 | |
| Sham-smoked | 35,873 | (18.5) | 19,021 | ( 9.8) |
| Unstimulated | 2,321 | | 2,321 | |
| Bench Control | 29,304 | (12.62) | 24,270 | (10.5) |

**Conditions:** 16 mice in smoked group
8 mice in sham-smoked and bench control

**Cigarette:** Kentucky Reference 1R1

**Exposure:** 3 Cigarettes, 2 times/day, 7 days

**Additional Information:** Spleens removed on day 7 and placed in culture with mitogen (25µg PEA, 2.0µg ConA) for 72 hrs. Pulsed for final 24 hrs. with 1.0µCi $^3$H-thymidine.

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Source: https://www.industrydocuments.ucsf.edu/docs/xndm0000

Table 25

## Effect of Acute Exposure (15 Days) to Smoke on the Response to Mitogenic Stimulation

| Group | $^3$H-Thymidine Incorporated (cpm) | | | |
|---|---|---|---|---|
| | Concanavalin A | | Phytohemagglutinin | |
| Unstimulated | 4,838 | (11.4) | 4,838 | (4.8) |
| Smoked | 55,319 | | 23,363 | |
| Unstimulated | 4,612 | (13.6) | 4,612 | (4.8) |
| Sham-smoked | 62,660 | | 22,074 | |
| Unstimulated | 4,852 | (13.0) | 4,852 | (4.4) |
| Bench Control | 63,266 | | 21,316 | |

**Conditions:** 16 mice in smoked group
8 mice in sham-smoked and bench control

**Cigarette:** Kentucky Reference 1R1

**Exposure:** 3 Cigarettes, 2 times/day, 15 days

**Additional Information:** Assay as indicated in Table 24.

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Source: https://www.industrydocuments.ucsf.edu/docs/xndm0000

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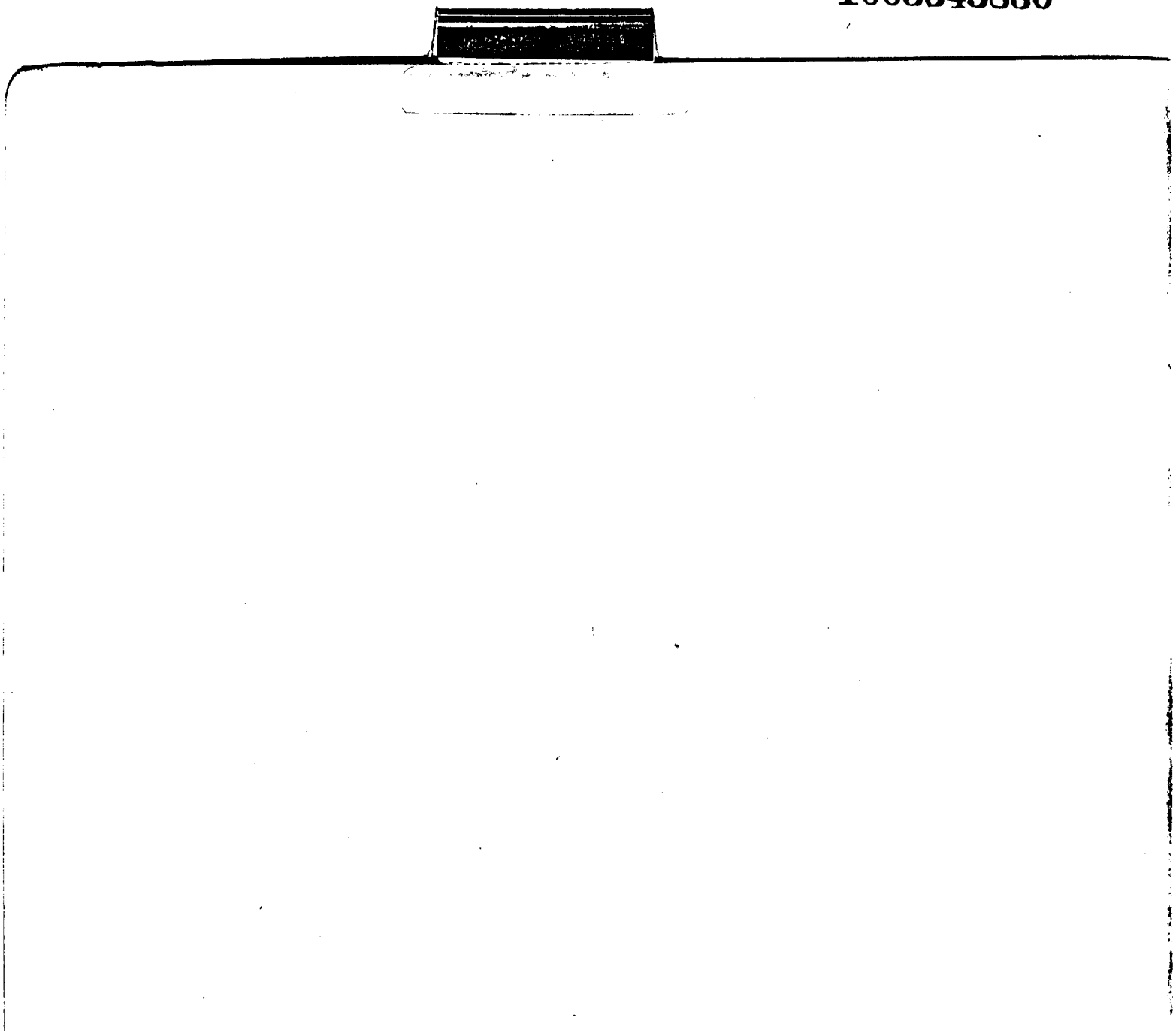

Source: https://www.industrydocuments.ucsf.edu/docs/xndm0000

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

July 16, 1975

Grant application No. 936R2

PULMONARY

To: The committee comprising Gardner, Sommers, Meier and Wyatt

Subject: Branislav Vidic, S.D., Georgetown University, D.C.
Second renewal application No. 936R2
"The Effect of Cigarette Smoke on Lung Metabolism"

History

A three year plan was approved by the SAB in October 1973 at a level of $36,256 for the period January 1, 1974 through December 31, 1974. Application No. 936R1 (first renewal year, i.e., 1975) requested $49,741, and was approved at a level of $40,000. In June 1975 Vidic received a supplemental of $4,800.

Request

Application No. 936R2 (second renewal) requests $56,736 for the period January 1, 1976 through December 31, 1976.

Documents submitted (attached)

1. Application dated June 20, 1975 (6 pages).
2. Progress Report No. 2 (10 pages).
3. Two manuscripts (submitted to Jour.Cell Biology and Journal of Morphology, respectively).
4. One reprint.

Comment

a. Salary for M. Hamosh, Ph.D. requested in this application will be used to support a replacement biochemist, if Project 2 of the P. Hamosh Program Project application (No. 1049) is approved.

b. As progress appears to be satisfactory, this request will be handled administratively and withheld from the bulky SAB agenda for September 1975, unless you object.

DS:wg
Atts.

D.S.

1003545831

Source: https://www.industrydocuments.ucsf.edu/docs/xmd0000

THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application For Renewal of Research Grant

(Use extra pages as needed)

First Renewal ☐ Second Renewal ☒ Date: June 20, 1975

1. Principal Investigator (give title and degrees):

Branislav Vidic, S.D., Professor of Anatomy, Department of Anatomy, Georgetown University School of Medicine and Dentistry

2. Institution & address:

Georgetown University School of Medicine and Dentistry, 3900 Resevoir Rd., N.W., Washington, D.C. 20007

3. Department(s) where research will be done or collaboration provided:

Department of Anatomy and Department of Physiology & Biophysics

4. Short title of study:

The Effect of Cigarette Smoke on Lung Metabolism

5. Proposed renewal date: January 1, 1976

6. How results to date have changed earlier specific research aims:

Because of technical delay in the initiation of smoking experiments, the project outlined in design II is slightly behind schedule. On the other hand, we have begun preparatory work of design III much ahead of the planned starting date.

7. How results to date have changed earlier working hypothesis:

The working hypothesis remains unchanged.

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Source: https://www.industrydocuments.ucsf.edu/docs/xndc0000

8. Any additional facilities now required? Describe briefly:

None

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

See Appended Page 1

10. Append outline of experimental protocol for ensuing year. See description of designs II and III

11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

See Appended Page 2

12. Summary progress report (append in standard form as separate document, unless recently submitted).

10035458 33

Source: https://www.industrydocuments.ucsf.edu/docs/xpdm0000

13. Budget for the coming year:

| A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested) | % time | Amount |
|---|---|---|
| Branislav Vidić, S.D. | 30 | |
| Paul Hamosh, M.D. | 10 | |
| Margit Hamosh, Ph.D. | 100 | 23,900* |
| Research Associate for electron microscopy (to be named) | 100 | 14,136* |

* Include 16.7% (1/1/76 - 6/30/76) & 20% (7/1/76 - 12/31/76) Fringe Benefits

Technical

Sub-Total for A 38,036

B. Consumable supplies (by major categories)

| | |
|---|---|
| Experimental animals | 3,850 |
| Chemicals for ultrastructural studies and photographic supplies | 2,200 |
| Radioactive isotopes | 1,100 |
| Chemicals for cellular culture and biochemical studies | 1,650 |
| Sub-Total for B | 8,800 |

C. Other expenses (itemize)

| | |
|---|---|
| Maintenance of equipment | 1,500 |
| Travel | 1,000 |
| Sub-Total for C | 2,500 |
| Running Total of A + B + C | 49,336 |

D. Permanent equipment (itemize)

None

Sub-Total for D

E. Indirect costs (15% of A+B+C) 7,400

O.K. PRB

Total request 56,736

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Source: https://www.industrydocuments.ucsf.edu/docs/xndm0000

14. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|---|---|---|---|
| Cellular and Immunological Basis of Pulmonary Diseases (co-investigator) | NHLI 3294-208 | $250.000 | 5/1/74- 4/30/79 |
| The effect of pulmonary blood flow on surfactant synthesis | Washington Heart Assoc 3282-752 | $10.350 | 1/1/75-31/12/75 |

PENDING OR PLANNED

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|---|---|---|---|
| | | | |

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Branislav Vidic, S.D.

Signature [signature] Date 6/20/75

Telephone 202 (Area Code) 625-7527 (Number) (Extension)

Checks payable to

Georgetown University

Mailing address for check: Sam A. Kimble
Georgetown University, 37th &
O Streets, N.W., Washington,
D.C. 20007

Responsible officer of institution

Typed Name Sam A. Kimble

Title Administr. of Sponsored Programs

Signature [signature] Date 7/3/75

Telephone 202 (Area Code) 625 - 3151 (Number) (Extension)

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Source: https://www.industrydocuments.ucsf.edu/docs/xndm0030

The year following, emphasis will be on projects outlined in designs II and III. While the biochemical studies remain in the hands of M. Hamosh, Ph.D., the most of the forthcoming technical and semi-technical involvement concerns the structural studies including: conventional transmission microscopy of isolated cells and intact lung pieces, scanning studies of isolated cell, and the auto-radiographic studies of cells in situ and isolated cells following administration of 3H palmitic acid. Furthermore, the principal investigator is planning to undertake major involvement in structural studies related to the Program Project, therefore we made the following redistribution in the salary category: Part of the biochemical investigations presently conducted by Y. Shechter, Ph.D., will be continued under the supervision of M. Hamosh, Ph.D., who is in charge of our biochemical laboratories. The funds made available will be conveyed toward hiring a competent electron microscopist, knowledgable in cytochemical techniques required for our studies: Dr. J.T. Wolosewick, University of Colorado at Boulder, Dr. Keith Porter Laboratory, Department of Mollecular, Cellular & Development Biology, Boulder, Colorado 80302.

1003545836

Source: https://www.industrydocuments.ucsf.edu/docs/xxxx0000

Vidić, B., and Tandler, B. Ultrastructure of the Secretory Cells of the Submucosal Glands in the Human Maxillary Sinus. submitted to J. of Morph. (see attached manuscript).

Vidić, B., Interaction of Membranous Phospholipid Layers During Coalescence Between Some Secretory Granules (Abstract accepted for Proceedings of the Tenth International Congress of Anatomists, Tokyo, Japan, August, 1975). (see attached manuscript).

Vidić, B., Hamosh, M., and Hamosh, P. Mucopolysacchrides in the Pulmonary Surfactant Hypophase. An Electron Microscopical Study of Rat Lungs Treated with Ruthemium Red. submitted to J. Cell Biol. (see attached manuscript).

Vidić, B., Hamosh, M., Blunda, M., and Hamosh, P. Synthesis of Surfactant as a Function of Ventilation (in preparation).

Vidić, B., Compton, S., Wrathall, J. and Hamosh, M. Synthesis of Surfactant by Isolated Type II Pneumocyte (in preparation).

Vidić, B., and Hand, A. (NIH, NIDR) Interaction of Membranes during the Coalescence among the Secretory Granules of Human Upper Respiratory Glands (in preparation).

Vidić, B., Secretory pathways in Rat Tracheal Goblet Cell. Mechanism of Secretory Granule Release (to be submitted to J. Cell Biol.).

Hamosh, M. and Hamosh, P. The Effect of Estrogen on the Lipoprotein Lipase Activity of Rat Adipose Tissue. J. Clin. Invest. 55: 1132, 1975

Hamosh, M. and Hand, A.R. Development of Secretory Activity in Rat Lingual Serous Glands. J. Dent. Res. 54: 168,1975

Hamosh, M. and Hamosh, P. Lipoprotein Lipase in Rat Lung, the Effect of Fasting. Biochim. Biophys. Acta. 380: 132, 1975

Hamosh, M., Yeager, H. Jr., Schechter, Y., Blunda, M., Vidic, B. and Hamosh, P. Lipoprotein Lipase in Rat Lung, The Effect of Hormones (in preparation)

1003545837

Source: https://www.industrydocuments.ucsf.edu/docs/[illegible]

## SUMMARY PROGRESS REPORT

Vidic, B., and Tandler, B. Ultrastructure of the Secretory Cells of the Submucosal Glands in the Human Mandible [illegible] submitted.

In our previous report we indicated changes concerning slightly delayed progress of designs I and II and early initiation of design III. Our group has been joined by J. Wrathall, Ph.D. and Miss S. Compton, B.S. from our Department. Their expertise in the tissue culture techniques enabled us to quickly advance our knowledge about the biological changes related to isolation, short-term, and long-term cultured type II pneumocyte. Results until now indicate that the isolation of type II cell from young postnatal rats following mincing of the lung, trypsin application and subsequent centrifugation of cell suspension over 2M sucrose is successful. We find that in early stages of culturing, type II cells deplete most of the lamellar bodies. This phenomenon is perhaps caused by osmotic changes, or by some additional conditions, in transferring the cell from in vivo to an in vitro situation. Furthermore, some of the most recent findings of early cultured type II cell demonstrate varying quantities of intracellular fat droplets, which are never observed in an in vivo condition. Our hypothesis supports the view that the fat droplets (see figures 1-4) at this stage of cell culture might represent the end-product of phospolipolytic enzyme activity upon the preexisting phospholipids contained with the lamellar bodies and/or mitochondria.

In order to test this hypothesis we plan to assay these cells for acid phosphatase activity, and possibly for phospholipase content according to biochemical procedures. Another aspect related to the cultured situation is the cellular response to corticosteroid treatment, a condition that is extensively studied in human, perinatal pulmonary pathology. Our data derived from the treatment of cells with different doses (0.1 mg/ml - 2.0 mg.ml) of dexamethasone suggest that the drug slightly increases the lecithin content inside the cell (see Tables I and II). These preliminary observations will be expanded in a systematic way to study the ratio of dipalmitoyllecithin (DPL) synthesis by type II cell as a function of hormonal influence in a "clean" and a nicotine-containing environment. It is also hoped that a better methodology will be established to test the surface tension activity of the extracellular medium.

[illegible] Dipalmitoyllecithin Dipalmitoyl in Rat Lung, the Effect of Hormones [illegible]

Our previous autoradiographic studies with 3H palmitic acid incorporation into DPL indicated a dependence of surfactant synthesis and/or excretion from the cell on the tidal volume of ventilation. Because in the first experiments, the time intervals at which the tissue samples were fixed for autoradiography were too distant, another extensive experiment was undertaken sometime early this year. In the latter experiment, the tissue samples were collected at 1, 3, and 5 minute intervals following the injection of the isotope.

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Source: https://www.industrydocuments.ucsf.edu/docs/xhdm0000

SUMMARY PROGRESS REPORT

Like the previous experiments, the amount of palmitic acid in type II cells was greater in hyperventilated than in normal-ventilated and still greater than in non-ventilated lungs. It was ascertained that the earliest appearance of labelled fatty acid on the alveolar cell surface of type II cell and/or on the surfactant layer of the alveolus occurs within 3 minutes after the injection of the label. In view of these findings we are projecting some other techniques, in addition to ones already in use, for projects I and II. Because information is needed on the nature of label in the alveolar space and in the continuous surfactant l ining layer at different time intervals, all of the future experiments will include the count of radioactive content from the lavaged lungs also. This additional information will provide a meaningful support to the analytical studies on the rate of surfactant synthesis as a function of ventilatory volume in control and experimentally "smoked" animals.

an

fat

In preparation for our studies on the effect of tobacco smoke on lung metabolism we have first investigated lung metabolism in normal rats. Since there is in general a lack of standardization of the methodology employed in the study of lung metabolism we have adapted the following methods to the study of lung metabolism in tissue slices:

1. Glucose Metabolism measured as the amount of U $^{14}C$ Glucose metabolized to $^{14}CO_2$ (aerobic metabolism), lactate (glycolysis) and incorporated into tissue lipid.
2. Protein Synthesis measured by the incorporation of labeled leucine into lung protein. cell (see Tables I and II). These preparations
3. Glucoprotein Synthesis measured by the incorporation of glucosamine.
4. Lipid and Phospholipid Synthesis measured by the uptake and esterification of labeled palmitate.

We have chosen the tissue slice method since it is the least time consuming and according to recent studies has an identical glucose metabolism as that of the perfused lung.

We have adapted the following analytical methods to the study of lung composition:

RNA, DNA, protein, lipid analysis (separation, identification and quantitation of the individual components of the major lipid classes-phospholipids and neutral glycerides).

We have at present completed 2 series of experiments in which rats were

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Source: https://www.industrydocuments.ucsf.edu/docs/xndm0000

exposed to the smoke of Kentucky reference cigarettes IAI or IRI. Exposure was twice daily, 7 days a week for 4 weeks. Each run contained 4 groups of 6-8 rats each:
Group I 1 cigarette twice daily
Group II,3 cigarettes twice daily
Group III control machine exposed
Group IV control- same batch of rats as Groups I-III, but never in contact with the smoking machine.
We are now processing our data for experiments with IAI cigarettes (the experiments with IRI cigarettes are still in progress). Preliminary experiments with IAI cigarettes indicate:
1. Palmitate incorporation and esterification was not affected by exposure to cigarette smoke.
2. There is a slight decrease in leucine incorporation into protein in the 3 cigarette exposed rats.
3. There is an increase in glucose oxydation after 1 or 3 cigarette exposure.
4. There is a marked increase in glucoprotein synthesis in rats exposed to 1 or 3 cigarettes.
There are slight differences between the two control groups, indicating that exposure of the animals to the smoking machine (without smoking) affects their metabolism.

We have initiated experiments on the biochemistry of the developing lung. These experiments are carried out in rabbits (late fetal, neonate and throughout the developmental period). The rabbits are a gift from Dr. Bellanti (Dep. of Pediatrics) who is studying the development of defense mechanisms in the lung. This study will therefore investigate if there is any correlation between "biochemical maturation" and development of immune competence in the lung.

We have continued our studies on lipoprotein lipase activity in the lung, with special emphasis on the effect of hormones known to affect the enzyme activity in other tissues. Our results indicate that of a number of hormones tested only dexamethasone caused a change in enzyme activity-stimulation. The increased demand for palmitic acid needed for the steriod stimulated surfactant synthesis could therefore be met by an increased uptake of triglyceride-fatty acids, which is regulated by



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lipoprotein lipase. The manuscript is now in preparation As part of this study on lipoprotein lipase activity we made the interesting observation that estrogen administration markedly decreases lipoprotein lipase activity in adipose tissue of male and of ovariectomized rats. (See appended reprint).

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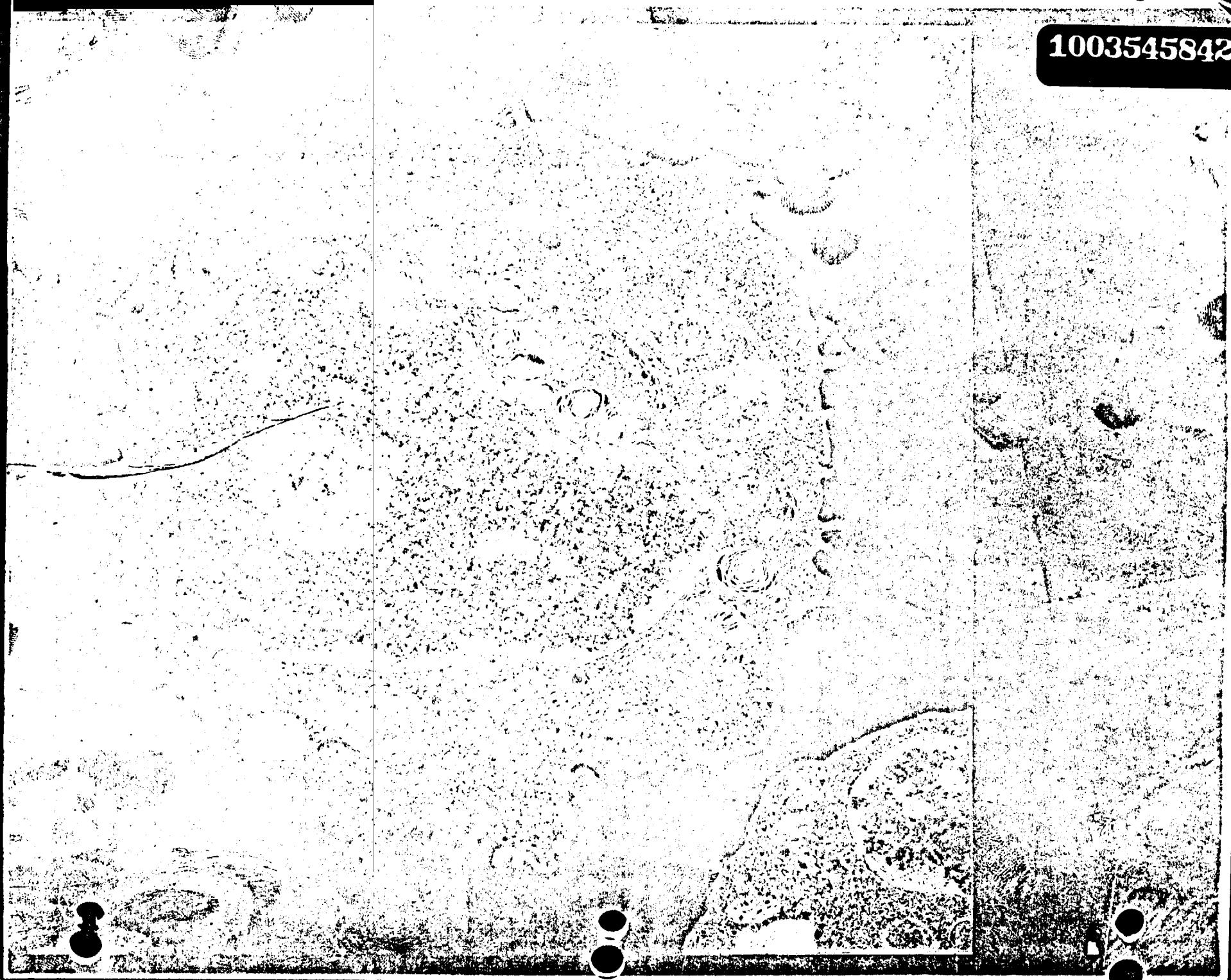

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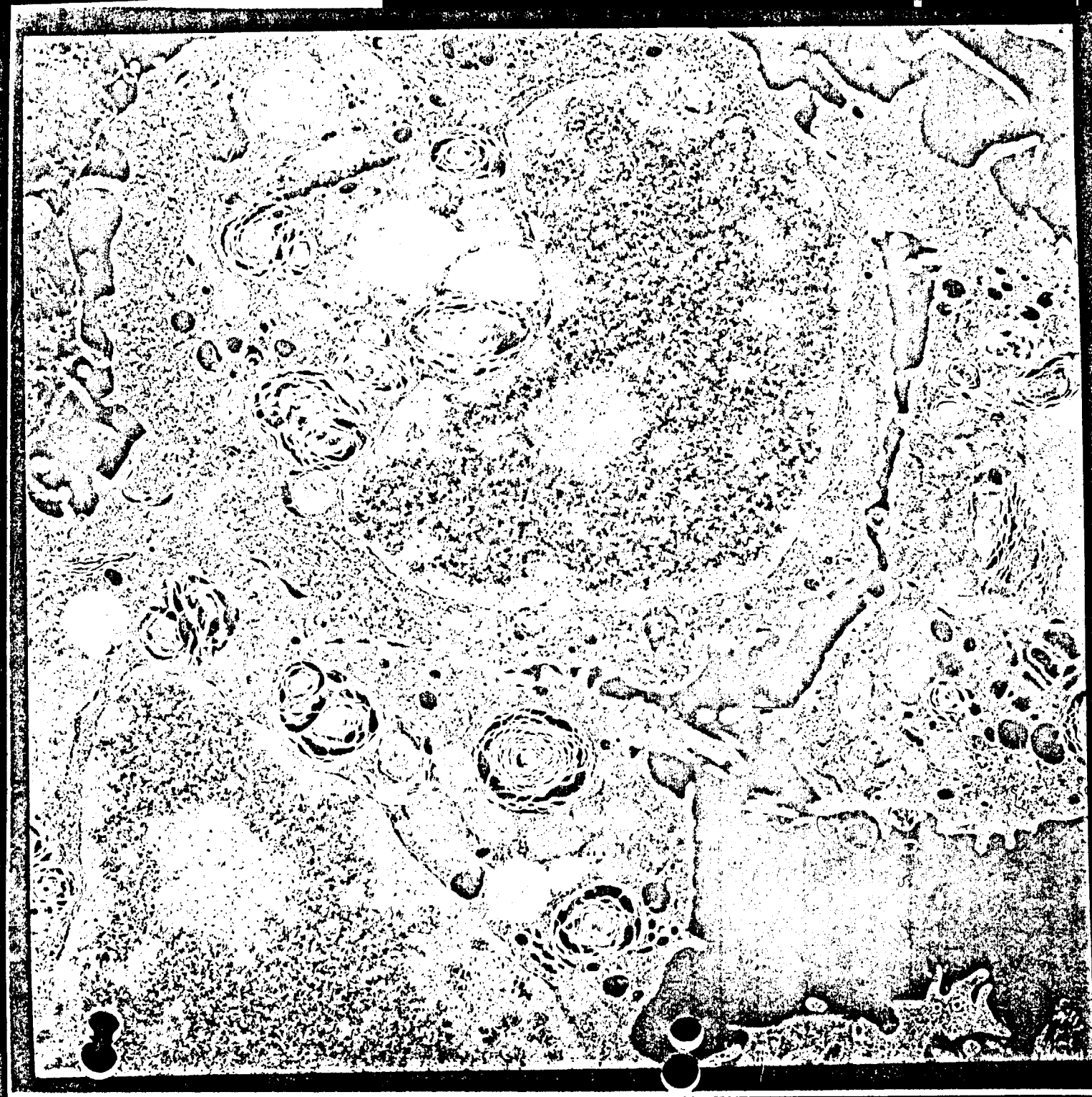

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CELLS

| | CONTROL | 0.1 MICROGRAMS DEXAMETHASONE | 2.0 MICROGRAMS DEXAMETHASONE |
|---|---|---|---|
| ORIGIN + LYSOLECITHIN | 2%<br>494 CPM | 16%<br>13383 CPM | 7%<br>1582 CPM |
| LECITHIN | 17%<br>5231 CPM | 38%<br>31783 CPM | 9%<br>2056 CPM |
| PE | 3%<br>814 CPM | 4%<br>3680 CPM | 3%<br>586 CPM |
| NEUTRAL FATS | 78%<br>22666 CPM | 42%<br>35045 CPM | 81%<br>18348 CPM |
| | 29205 CPM TOTAL | 83891 CPM TOTAL | 22572 CPM TOTAL |

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MEDIA

| | CONTROL | 0.1 MICROGRAMS DEXAMETHASONE | 2.0 MICROGRAMS DEXAMETHASONE |
|---|---|---|---|
| ORIGIN + LYSOLECITHIN | 0%<br>0 CPM | 1%<br>1671 CPM | 1%<br>57 CPM |
| LECITHIN | 0 %<br>0 CPM | 1%<br>1114 CPM | 1%<br>171 CPM |
| PE | 0%<br>0 CPM | 2.6%<br>14479 CPM | 9.4%<br>4459 CPM |
| NEUTRAL FATS | 100%<br>2887 CPM | 97%<br>536281 CPM | 90%<br>42687 CPM |
| | 2887 CPM TOTAL | 553545 CPM TOTAL | 47374 CPM TOTAL |

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ABSTRACT:

The coalescence between secretory granules facilitates the extrusion of a viscous material from the acinar cells into the ductal system. In the present report, the interaction of granular membranes during the coalescence was evaluated in the human mucous cells situated within the submucosal layer surrounding the maxillary sinus. In contrast, the serous granules of the other cells do not engage in any aspect of coalescence prior to their release into the lumen. The first ultrastructural indication of the coalescence is the transformation of phospholipid layers from both juxtaposed membranes into the pentalamellar profile. Similar to the reactions of the membranes investigated during the intralysosomal digestion, the middle phospholipid zone resulting from either apposition or interdigitation of two molecular layers is wider than the peripheral osmiophilic segments. Subsequent lysis of the modified membrane proceeds from only one side of the pentalamellar arrangement. This decomposition suggests that only certain secretory granules contain, in addition to acidic mucosubstances, the lipolytic enzymes capable of analysis of phospholipid layers. Although the nature of the triggering mechanism which activates these enzymes is not thoroughly understood, it appears that the displacement of the phospholipid chains proceeds first along the ipsilateral membrane. After the ipsilateral membrane is completely digested, the remaining membrane between two granules shows a typical unit structure. The further hydrolysis of such a membrane preceeds the fusion of granular matrices. In addition, the broken ends of both pairs of phospholipid layers reorganize to become continuous with their respective internal and external counterparts. The ultrastructural evidence presented herein indicates that the interaction of biological membranes during the coalescence of secretory granules is similar to processes suggested in pinocytosis, the formation of secondary lysosomes, the condensation of secretory material adjacent to the Golgi apparatus, and many others. (Supported by Grant from The Council for Tobacco Research - USA, Inc.)

--1) Vidic, B. (1975). Proceedings of the Tenth International Congress of Anatomists, Tokyo, Japan, August 24-30. "Interaction of membranous phospholipid layers during coalescence between some secretory granules."

From the Department of Anatomy, Georgetown University School of Medicine, Washington, D.C.

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J. Cell Biol

# MUCOPOLYSACCHARIDES IN THE PULMONARY SURFACTANT HYPOPHASE. AN ELECTRON MICROSPICAL STUDY OF RAT LUNGS TREATED WITH RUTHENIUM RED. *

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## Abstract

The alveoli of the lung are coated by surfactant, composed of phospholipids, proteins and mucopolysacchrides. Morphologically, it consists of an epiphase and a hypophase. The location of mucopolysacchrides in the surfactant has been identified through the use of Ruthenium Red (RR), a stain specific for mucoid substances. Electron microscopic analysis showed RR-positive material in association with the external surface of "tubular myelin", a component of surfactant hypophase. These observations support the hypothesis that the surfactant system consists of sliding layers of phospholipid, separated by mucopolysacchrides and protein.

## Introduction

The surfactant system, an osmiophilic superficial layer covering the alveolar surfaces, is usually subdivided into two layers. There is a thin superficial layer called the epiphase, and a layer of varying thickness, found between the cell membrane and the epiphase, called the "base layer" (4) or hypophase (12). There is general agreement that the epiphase consists of dipalmitoyl

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lecithin (DPL) and other components of the lung surfactant system. The hypophase, however, is morphologically complex, and its chemical composition is not well understood. The hypophase is irregular in thickness. In well preserved specimens, it seems to fill the space between the smooth epiphase layer and the irregular cell surface. It is amorphous in appearance, with a varying amount of vesicular profiles, and in some places it forms intricate patterns called "tubular myelin".

Histochemical and biochemical studies of lung washings show, in addition to phospholipids and mucopolysacchrides, significant amounts of protein and glucoprotein. Since these substances do not form mono- or bi- layers, as does DPL, they probably originate from the hypophase. The presence of mucopolysacchrides in the hypophase is probably essential for the proper function of the surfactant system. In the present study, Ruthenium Red (RR) was used in an effort to illustrate the spatial relationship of mucopolysacchrides to other components of the surfactant system.

Materials and Methods

Adult, healthy Sprague-Dawley rats of both sexes were injected intraperitoneally with lethal doses of Pentobarbital. Following thoracolaparotomy and removal of the diaphragm along the costal attachments, pieces of lung were excised from both

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lower lobes. Some samples were double-fixed by immersion, whereas others were first immersed for two hours at 4°C in a solution (8) made of equal quantities of glutaraldehyde-formaldehyde stock mixture (6), 0.1M sodium cacodylate buffer pH 7.4, and RR stock solution (1,500 ppm in water). A brief rinse of the tissue in cold buffer was followed by a second fixation and an additional application of RR (equal quantities of a 5% aqueous solution of osmium tetroxide, 0.1M sodium cacodylate buffer pH 7.4, and RR stock solution) at 4°C for one hour. In some animals the trachea was transected and connected to a small rodent respirator by a cannula. A vascular cannula was introduced through the right ventricle and connected to a peristaltic pump. Unimpeded venous outflow was secured by wide excision of the left auricle. The specimen was suspended from a force transducer in a thermal chamber at 37°C. Both lungs were ventilated in the chamber one to three times per minute at one ml tidal volume. They were then perfused for one to two hours with the solution of aldehydes and RR, prior to the removal of samples from both lower lobes. In some instances, the perfusion was continued using first the buffer solution and then the second fixative. All specimens were stained en block at room temperature for one hour in 1% uranyl acetate in maleate buffer pH 6.0, dehydrated and embedded in Epon (7). Thick sections (0.5 to 1.0μ) were used to evaluate the penetration of RR into the blocks. Thin sections were treated with heavy metal salts (14,15) and examined in an AEI-EM 801 electron microscope.

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Results and Discussion

RR does not readily penetrate the cellular interior. To the small extent that it does penetrate, it is bound to the lipid droplets or the internal aspects of the endoplasmic reticulum membranes. Some investigators (5) attribute this location to cross-bindings from the hexavalent RR to the negatively charged part of the juxtaposed phospholipid layers comprising endoplasmic reticulum. When stained by RR, the cysternal configuration appears as a pentalamellar form in thin sections (5). However, most RR-positive material is found outside the cell, in the cellular coat, where it is attracted by polyanionic complexes of acidic syalo- and/or sulfo- mucinous molecules. These molecular complexes are probably made up of polypeptides and polysacchrides which are bound in varying proportions. Biological membranes do not provide an adequate number of anionic arms for binding a hexavalent cation such as RR (8). Thus, when RR is used alone, phospholipid mono- or bi- layers are not stained.

The difficultues encountered in labeling the inside of the alveoli by intra-vascular administration of RR, were due to a lack of penetration by the dye across the pulmonary endothelium. The surfactant system (4) was almost entirely destroyed during fixation by immersion. For these reasons, an attempt was made to first use perfusion fixation with a solution of aldehyde-RR, and then to immerse the speciman into RR-containing fixative. Although it was impossible to preserve the entire surfactant

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layer within any single alveolus, there were sufficient locations where the epiphase and hypophase were satisfactorily preserved.

Figure 2 shows the surfactant epiphase, a monolayer coating a type II alveolar cell. An indentation in the adjacent cell is filled with an amorphous material, which has a vesicular appearance toward the monolayer. A similar area of hypophase is shown in Figure 3. The proximity of the vesicles to the monolayer is remarkable. It immediately raises the unresolved question: Is the hypophase a precursor of the monolayer or a product of its degradation?

Figure 4 shows some "tubular myelin" coiled up in the hypophase. This configuration might either represent a just extruded lamellar body or some worn out surfactant debris. Phospholipids do form these configurations in aqueous solution regardless of their functional viability. Figure 5 shows all forms of hypophase, and might support the evidence that the hypophase is synthesized de novo, because of the presence of active transporting vesicles. Endo- and pinocytosis by type II cells occurs rarely, if at all. Sometimes the entire hypophase consists of "tubular myelin" (Fig. 6). These networks are composed of typical trilaminar membranes with a diameter of about 45 Å that intersect at approximately right angles. The resulting gridholes range in diameter from 313 Å to 571 Å. This range is compatible with the values obtained for lattice-like membranes of the newborn rabbit after pharyngeal deposition of surfactant (11).

Assuming that three distinctly identifiable components of

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the hypophase (amorphous, vesicular, "tubular myelin") are of similar biochemical composition, their appearance indicates a complex physico-chemical environment and not a simple aqueous phase (11).

Surfactant treated with fixatives containing RR. The perfusion-fixation technique precipitated RR only on the cellular coats of endothelial cells, as far centrifugally as the tight junctions. The immersion-fixation method facilitated binding of RR to cellular coats of epithelial cells as far as their tight junctions. In both instances, the cellular coats bind RR molecules (Figs. 1,7) along an approximately 320 Å wide zone, for comparison see (1). Contrary to previous findings (10), no intracellular binding sites for RR were found in this study, with the exception of lipid globules inside the macrophage, and cytoplasmic inclusions of the mast cell.

Two distinct parts of the pulmonary surfactant are repeatedly observed to bind a great quantity of RR molecules: 1) the zone immediatly adjacent to the monolayer (Fig. 8); and 2) the lattice-like membranous networks of the surfactant hypophase (Figs. 9, 10). In the former location, RR reveals a "fuzzy" layer with a diameter similar to that of the cellular coat of either the endothelial or the epithelial cells. Some of the vesicles in the hypophase are also stained with RR, but only externally.

In the lattice-arranged membranes, the hexavalent cation binds heavily along the boundaries of the gridholes. In this

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instance, the labelled complexes seem to be associated with the external faces of the crossing membranes, while the membranous interior remains label-free. Consequently, the membrane itself, in this preparation, appears as a negatively stained image with RR-positive material @ 90 to 150 Å in diameter, paralleling it on both sides. The opacity of this material is particularly pronounced at the crossing points of perpendicularly intercepting membranes. These findings support some speculations on the composition and organization of the surfactant system (2). The surfactant epiphase is a monolayer of phospholipid and has its chain (hydrophobic) poles oriented toward the alveolar surface and its hydrophilic poles toward the cell surface. Therefore, since RR reacts only with the hydrophilic phase, it stains only the inside of the monolayer (Fig. 8). The external cell coats are well-stained only where not protected by surfactant. Even more significant is the presence of RR-positive material, presumable mucopolysacchrides, at the cross-over points of the lattice work. Assuming that the surfactant system consists of several layers of surface active material which glide on each other during respiration (13), it is difficult to visualize such motion without some other material interposed between the adjacent polar surfaces. It was suggested that the surfactant system consists of layers of saturated DPL, stabalized by Ca++ and Na+ ions (9), polysacchrides, sialo and/or other mucins (3), and other phospholipids. The location of

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RR-positive material between layers of phospholipids or separating these layers from the cell surface lends considerable credence to the above speculations.

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FOOTNOTE

* This investigation was supported by Grants from the Washington Heart Association and The Council for Tobacco Research, U.S.A.

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PLATE I

EXPLANATION OF FIGURES

1.In this view the depth of penetration by RR through the capillary endothelium and intercellular spaces (S) is demonstrated. The binding of RR to the glucoproteins of the cellular coat is apparent. Also shown is the labelling of some pinocytotic figures (arrowhead). The basal lamina supporting the endothelial cells is indicated by an arrow. Double fixation with RR by way of pulmonary artery, uranyl acetate and lead citrate stain. X 18,900

2.A close relationship between the surfactant monolayer (arrow) and the villar projections of type II pneumocyte (P) is demonstrated in this section. In an indented part of the alveolus, the amorphous (A) and vesicular (arrowhead) layers of the hypophase are also shown. Uranyl acetate and lead citrate stain. X 35,000

3.Another indentation of the alveolus between the endothelial cells (E) is primarily occupied by the amorphous phase (A) of the surfactant. This material is covered toward the alveolar lumen (L) by a discrete layer of vesicle-like profiles (arrow) and the surfactant monolayer (arrowhead). Uranyl acetate and lead citrate stain. X 67,000

4.The surfactant hypophase shows, in addition to the amorphous material (A) and the vesicular layer (arrows), a large myelin-like figure. Note the distribution of vesicles in relation to the figure. The surfactant monolayer is indicated by an arrowhead. Urnnyl acetate and lead citrate stain. X 24,000

5.From the endothelial cells (E) toward the alveolar lumen (L), the surfactant hypophase consists of the following layers: amorphous (A); vesicular (V); and lattice-arranged membranes (M). In similar sections the vesicular layer appears as a transitional material between the amorphous and membranous phases. Note also the structure of the

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PLATE I (Continued)

material contained in several endothelial transporting vesicles (arrows) in comparision to the amorphous phase. Uranyl acetate and lead citrate stain. X 45,000

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PLATE II

EXPLANATION OF FIGURES

6. In some instances, as is demonstrated in this section, the entire depth of the surfactant hypophase is represented by the lattice-arranged membranes measuring @ 2.4μ in thickness. The surfactant monolayer is indicated by an arrowhead, and the basal lamina supporting the endothelial cells (E) by an arrow. Uranyl acetate and lead citrate stain. X 17,000

7. Once the surfactant monolayer becomes disrupted by the fixation procedure, RR binds extensively to the cellular coats, covering the surface of both types of pneumocytes. No intracellular organelle of type II pneumocyte shows the reaction product. Double fixation with RR by immersion. X 8,000

8. Because of the immersion fixation procedure, the surfactant monolayer (arrowhead), although still properly oriented, is dis-associated in this section from the surface of type II pneumocytes (arrow). The binding sites of RR correspond to the hydrophilic poles of the monolayer and the cellular coat, respectively. Double fixation with RR by immersion, uranyl acetate and lead citrate stain. X 23,000

9. Conventional electron microscopy reveals certain material, similar to that described for the cellular coat, in association with the lattice-arranged membranes of the hypophase. This material is especially concentrated at the crossing points between membranes (arrow). Uranyl acetate and lead citrate stain. X 126,000

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PLATE II (Continued)

10. RR binds strongly to the opaque material associated with the lattice-arranged membranes of the hypophase. In this instance, the crossing membranous bilayers appear as negative images. Double fixation with RR by immersion, uranyl acetate and lead citrate stain. X 150,000

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FOOTNOTE

*This study was supported in part by Grants from the Washington Heart Association, the Council for Tobacco Research, U.S.A., and by N.I.H. Grant 5S01FR05335-12.

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J. Morph.

# Ultrastructure of the Secretory Cells of the Submucosal Glands in the Human Maxillary Sinus*

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Number of Figures: 18

Running head: Submucosal Glands of Human Maxillary Sinus

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Ultrastructure of the Secretory Cells of the Submucosal Glands in the Human Maxillary Sinus.

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ABSTRACT. Tissue samples obtained from the lateral wall of the maxillary sinuses of five patients were examined by light microscopical, histochemical, and ultrastructural techniques. Submucosal glands were tubulo-alveolar mixed glands. The acini consisted of either all serous or all mucous cells, or of a mixture of both cell types. Serous granules were stained by toluidine blue or by hematoxylin and eosin, but showed little or no reaction with the PAS-reagent or with Alcian blue. Mucous granules were pale in toluidine blue or H and E preparations, and consisted primarily of acid mucosubstances, as demonstrated by their staining reaction with PAS and Alcian blue. At the electron microscope level, the serous granules were either homogeneously dense, or showed a substructure consisting of at least two layers of distinctly different electron-opacity. Typical mucous droplets consisted of a fibillar network dispersed in a translucent matrix. A second secretory product was present in the mucous cells in the form of elongated, membrane-bounded structures containing numerous, parallel filaments, which measured about 55Å in diameter. The mucous droplets and the filamentous bodies appear to arise from the opposite faces of the Golgi complex in the mucous cells. The filamentous bodies showed a pronounced tendency to fuse with the mucous droplets. All acini were surrounded by a well-defined myo-epithelial layer and contained intercellular nerve terminals.

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Many different exocrine glands are present in the submucosa of the nasal passages. Of these, only Bowman's glands (Frisch, '67; Seifert, '71b; Breipohl, '72), anterior nasal glands (Kerjaschki, '74), and vomeronasal glands (Seifert, '71a; Tandler and Bojsen-Møller, '74) have been examined by electron microscopy. In the maxillary sinuses, the lateral nasal (Steno's) gland (Moe and Bojsen-Møller, '71; Vidić and Taylor, '72) and the maxillary gland of the rat (Vidić and Greditzer, '71; Vidić, '73) have received some attention, but the dispersed mixed glands underlying the human sinus epithelium have been largely overlooked.

According to Sappey (1889) the submucosal glands are distributed throughout all of the walls of the sinus cavity. They are described as pleomorphic with respect to their size and acinar configuration. Although the submucosal glands are abundant and may make a significant contribution to nasal secretions, they have been virtually ignored since Sappey's time. Most modern textbooks of otorhinolaryngology acknowledge that there are such glands in the sinus submucosa, but give no histological description of these organs despite their possible role in nasal function.

The present article describes the histology, histochemistry and ultrastructure of these submucosal glands in the human maxillary sinus.

## MATERIALS AND METHODS

Tissue specimens were obtained by surgery from five adult patients at the District of Columbia General Hospital under the auspices of the Department of Otolaryngology.** The ablation of soft tissues from the maxillary sinus, was undertaken in each

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instance, because inverted papillomas originating from the medial wall of the sinus interferred with the ostium or with the communication of the sinus with the middle nasal meatus. Following general anesthesia and a local application of neosynephrine, the sinuses were perforated through the anterior wall from the oral vestibule, and several samples of grossly normal lining were removed from the lateral wall of the sinus. Samples were fixed for two hours at 4°C in a 3 to 6% solution of glutaraldehyde-paraformaldehyde (Ito and Karnovsky, '68) buffered to pH 7.2 with 0.1 M sodium cacodylate. The samples were then washed in buffer for 24 hours, postfixed for two hours at 4°C in a 2% aqueous solution of osmium tetroxide, stained in block for one hour with 1% uranyl acetate buffered with maleic acid to pH 6.0, dehydrated, and embedded in Epon. Thin sections were stained with heavy metal salts (Watson, '58; Venable and Coggeshall, '65) and observed in an AEI-EM 801 electron microscope.

For light microscopy, epoxy sections 0.5-1.0 mμ thick were stained with toluidine blue (Pearse, '68). A portion of each tissue specimen was fixed in formalin, embedded in paraffin, and sectioned at 5 mμ. These sections were stained with hematoxylin and eosin, periodic acid-Schiff and hematoxylin, or Alcian blue and nuclear fast red, and studied with a Leitz microscope.

## OBSERVATIONS

Light microscopy. The submucosa of the human maxillary sinus contained dispersed exocrine glands of the tubuloalveolar type (figs. 1, 2). Both serous and mucous cells were present in the glandular acini. In some acini, these cell types were intermingled, so that

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both serous and mucous cells bordered on the same lumen. Other acini consisted entirely of either mucous or serous cells. Mucous acini were sometimes capped by a serous demilune. Flattened myoepithelial cells were distinguishable at the acinar perimeter.

Mucous cells were characterized by a pale cytoplasm and a flattened, basally located nucleus. These cells contained abundant mucosubstances (pH range from 0.5-4.5), as demonstrated by their strong staining by the periodic acid-Schiff and Alcian blue procedures. The serous cells had a rather basophilic cytoplasm and a round nucleus usually situated in the basal third of the cell. These cells were only weakly PAS-positive, and showed no reactivity towards Alcian blue. Serous demilune cells were flatter than their acinar counterpart, but showed the same staining properties.

Electron microscopy. In serous acini or in mucous acini lacking serous demilunes, the secretory cells had the same general shape--they were columnar to cuboidal and somewhat narrower at their apex than at their base. Both serous and mucous cells bore microvilli on their apical surface, with those on the serous cells showing an obvious filamentous core. The acinar lumina ran an irregular course, so that the same lumen often was intersected several times in the plane of a single section. Adjacent cells demonstrated a considerable degree of lateral interdigitation. Basal folds were often present on the basal surface of the secretory cells, and these cells frequently rested on a myoepithelial cell or on several myoepithelial processes. Serous demilune cells had the same morphological features as the serous acinar cells, but were considerably flatter.

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The serous cells possessed all of the morphological hallmarks

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usually associated with serous-type cells, viz., an extensive granular endoplasmic reticulum, prominent Golgi complexes, abundant mitochondria, and numerous membrane-bounded secretory granules. The serous granules in the submucosal gland cells assumed a variety of appearances based on the density and distribution of their content (figs. 3, 4, 5, 6, 7). The most typical granules were moderately dense and homogeneous in appearance. In other cells, the granules often consisted of a light central region surrounded by a halo of substantially denser material. Certain other granules had the same arrangement of light and dark zones, but the difference in density between the two components was less pronounced. Furthermore, a distinct dark band served to separate the two zones from each other. In still other cells, the light and dark areas of the granule matrix were reversed so that the center of the granules was the darkest portion, with the periphery being lighter. Whatever their internal organization, the serous granules appeared to be liberated into the acinar lumen by a typical merocrine process (fig. 8).

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The most outstanding feature of the mucous cells was the abundance of mucous droplets that almost completely filled the supranuclear cytoplasm. Only a few scattered organelles were evident in the interstices between the closely-packed droplets. Typical mucous droplets were large (about 2 mμ in diameter), membrane-bounded, and showed a marked propensity for lateral fusion with neighboring droplets. They consisted of finely fibrillar material disposed in an irregularly reticulate pattern within a low-density matrix. The packing of the fibrillar meshwork appeared to be related to the degree of maturity of the droplets, being looser in

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the less mature droplets. In some mucous cells, the droplets contained a fairly dense spherule, which often was eccentrically placed (figs. 9, 10, 11). A second type of secretory granule, which tended to be elongated in shape rather than round, was present within virtually all mucous cells. These membrane-delimited bodies consisted of numerous parallel filaments (fig. 12), each measuring approximately 55Å in diameter, suspended in a low-density matrix. Viewed in transverse-section, the filaments appeared as dots (fig. 13). Small dense structures resembling lipid droplets were frequently scattered among the fibrils. Occasionally, these structures attained substantial size, and their resemblance to lipid was correspondingly enhanced (fig. 14). The filamentous bodies also demonstrated a proclivity for fusion with mucous-droplets (fig. 15); obvious fusion figures were often observed.

Both the mucous droplets and the filamentous bodies appeared to arise in association with the selfsame Golgi complex. On one face of this organelle, that associated with mucous droplets, the outermost cisternae appeared to be dilated, and contained finely filamentous material matching that in the droplets. On the opposite face of the Golgi complex, that facing the filamentous bodies, the cisternae contained fibrillar material resembling that in the bodies (fig. 16).

Nerve terminals (fig. 17) were often observed in an intercellular position. These frequently contained mixed populations of vesicles--large dense-cored vesicles and small apparently empty vesicles. Myoepithelial cells were typical in appearance, containing numerous myofilaments (fig. 18) and showing many pinocytotic vesicles at their surfaces.

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## DISCUSSION

Based on their staining reactions and on their ultrastructure, the acini of the submucosal glands of the human maxillary sinuses are seen to consist of either serous or mucous cells. These cells bear a strong resemblance to their counterparts in a variety of human salivary glands. Rather than being homogeneous structures, the serous secretory granules in the submucosal glands display a striking variability in their internal organization. While this variability in granule appearance within individual cells may be due in part to a maturational sequence, clear differences in granules are apparent in neighboring cells. Thus, in one cell the granules may have a dense rim with a light central zone, while in an adjacent cell the granules may have a light rim with a dense central zone, not to mention several other possible configurations. These differences in appearance suggest that even contiguous serous cells in submucosal glands may be producing somewhat different secretory products. A similar situation occurs in the serous cells of the human submaxillary gland (Tandler and Erlandson, '72). In the hamster submandibular gland, the appearance of the seromucous cells seems to be sex-linked (Dorey and Bhoola, '72). In the female hamster, these granules show a moderately-dense cortical region surrounding a pale inner area, while in the male the situation is reversed.

It has become apparent in the last few years that secretory granules with obvious substructure occur with far greater frequency than do granules with a uniform matrix; some of the manifold

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patterns assumed by secretory granules have been catalogued by Tandler and MacCallum ('72). The significance of matrix zonation in serous-type granules is not clearly understood. Digestion of thin sections with pronase has demonstrated that the complex organization of granules in lingual granules of the salamander is due to ordered disposition of the protein and mucosubstance components of the granule matrix (Zylberberg, '73). Similarly, histochemical tests carried out on thin sections have shown that acid mucosubstances are restricted to the electron-lucent rim of serous granules in the parotid and sublingual glands of the Mongolian gerbil (Ichikawa and Ichikawa, '74). The question has been posed whether or not this sequestration of stainable components in secretory granules is a reflection of the presence of discrete aggregates of different enzymes within individual granules (Tandler and Erlandson, '72). This question can be answered only after granules with a well-defined substructure are successfully isolated and analyzed.

The finely-fibrillar reticulate appearance of the content of mucous droplets, in the maxillary sinus submucosal glands, is similar to that of droplets in mucous and seromucous cells in different types of salivary glands; the stained material in the secretion of the latter organs has been suggested to be glycoproteins (Gallagher, Marsden and Robards, '69). In the submucosal glands, the mucous droplets show little of the range in density found in human labial salivary glands (Tandler et al., '69) or of the staining variations in submucosal glands in the human tracheo-bronchial tree (Lamb and Reid, '69). Occasional clusters of these droplets, however, may

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contain a dense spherule, an indication that there may be some differences in their composition. The submucosal mucous droplets show a strong tendency to fuse, producing relatively large, irregular, membrane-bounded aggregates of mucus. This process has been implicated in the extrusion of mucous secretions in the rat sublingual gland (Kim, Nasjleti and Han, '72).

In addition to mucous droplets, the mucous cells consistently contain at least a few, and sometimes many, filamentous bodies. These are identical in morphology to certain cytoplasmic structures found in mucous cells of human sublingual and submandibular glands (Tandler, unpublished observations) and of human palatine salivary glands (Shimono et al., '73). They also resemble the duplex inclusions described in human labial salivary glands (Tandler et al., '69) and the Biondi-like inclusions found in the human iris (Ringvold, '74), although the lipid component usually is less prominent in the submucosal gland filamentous bodies. Similar structures are present in mucous cells of the human endocervix, where it has been claimed (without any direct evidence) that they arise by transformation of ordinary mucous droplets by an irreversible process (Philipp, '72). From our observations, it seems obvious that the filamentous bodies represent a second type of secretion produced by the mucous cells of the submucosal glands of the maxillary sinus. The Golgi complex in these cells appears to simultaneously produce two distinct secretory products from its two faces; on one side, mucous droplets; on the other, filamentous bodies. The production of two discrete secretory products from the same Golgi complex is not without precedent; a similar finding has been documented in

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polymorphonuclear leukocytes, where both specific and azurophil granules arise in association with the Golgi complex, although in this case, the two secretory products are produced at different stages in development of the cells (Bainton and Farquhar, '66).

According to Philipp ('72), the filamentous bodies remain in endocervical cells when mucous is extruded. The ostensible absence of filamentous material in the gland lumen may be explained by our finding of fusion between filamentous bodies and mucous droplets, wherein the filaments appear to depolymerize. As a consequence, the biochemical components of the filaments are present within modified mucous droplets, albeit in a no longer recognizable form, and are available for release. The mechanism of filament depolymerization is unknown. It could be based on exposure to a change in pH of the secretory droplet as a result of fusion, or on exposure to enzymatic activity within the mucous.

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FOOTNOTE

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## FOOTNOTE

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PLATE 1

EXPLANATION OF FIGURES

1 This photomicrograph of the submucosal layer from the maxillary sinus demonstrates the general arrangement of serous (S) and mucous (M) secretory cells in several acini. The dark cells at the acinar periphery are myoepithelial cells (arrows). Toluidine blue. X 675.

2 Several acini composed entirely of either serous (S) or of mucous (M) secretory cells are indicated in this section. In addition, three acini (arrows) converge toward a common duct. Periodic acid-Schiff and hematoxylin. X 675.

3 An electron micrograph illustrating the diverse appearance of secretory granules in several adjoined cells. Note the relatively uniform ultrastructure of secretory granules within any individual acinar cell. Acinar lumina (L) are also shown. X 8,000.

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## PLATE 2

### EXPLANATION OF FIGURES

4, 5, 6, 7 These micrographs illustrate the various appearances assumed by secretory granules (G) in different serous cells. Some granules (fig. 4) contain a homogeneous electron-opaque substance. The other granules show two substances of distinctly different structure and opacity. Fig. 4, X 18,400; Fig. 5, X 22,500; Fig. 6, X 17,000; Fig. 7, X 48,000.

8 A serous granule (G) in the apical cytoplasm is closely apposed to the plasmalemma (arrow), suggesting initiation of membrane fusion preceding the extrusion of secretory material into the acinar lumen (L). X 80,000.

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# PLATE 3

## EXPLANATION OF FIGURES

9 The Golgi region of a mucous cell. Many of the droplets surrounding the Golgi apparatus (arrowhead) are in various stages of coalescence (arrows). X 20,000.

10 The internal structure of a mucous droplet. The granular membrane is indicated by arrow. X 125,000.

11 Inclusions of electron-opaque material (arrowheads) are found in some mucous granules. X 43,000.

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# PLATE 4

## EXPLANATION OF FIGURES

12 In this mucous cell, two types of secretory products are demonstrated. Typical mucous droplets (G) contain electron-opaque fibrils arranged in a network-like pattern. The other type of granule (arrows) contains parallel filaments. Small droplets, presumably lipid in character, are sometimes present in the filamentous bodies. X 18,000.

13 Filaments are demonstrated in perpendicular (P) and oblique (O) sections in the same secretory droplet. Possible initial fusion of this granule with an ordinary mucous droplet is indicated by the arrow. X 80,000.

14 Several filamentous bodies are present in this mucous cell. A large droplet (D) is bounded by the same membrane (arrow) that delimits one of the filamentous bodies. This presence within these bodies of lipid droplets of varying sizes is a common occurrence in the submucosal gland mucous cells. X 32,000.

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## PLATE 5

### EXPLANATION OF FIGURES

15 During the coalescence of a typical mucous droplet (G) with a filamentous granule (F), the interface (arrow) between their respective contents is clear. Subsequently the filaments disintegrate. This breakdown of filaments must precede the extrusion of secretory material, since in no instance was a filamentous granule observed exiting from the cell into the acinar lumen. X 64,000.

16 Transfer of secretory material from the Golgi apparatus to the secretory granules occurs in two directions. On one side of the apparatus the cisternae contain a material similar in ultrastructure (arrows) to that found in ordinary mucous droplets. Cisternae along the opposite face of the apparatus, contain material (arrowheads) similar in appearance to the filaments. X 64,000.

17 This intercellular nerve terminal is characterized by a mixed population of vesicles, with some large dense-cored vesicles and, smaller electron-translucent vesicles. In addition, the terminals may contain neurotubules, bundles of microfilaments, and mitochondria. The membrane of this terminal is indicated by an arrow; the plasmalemma of the adjacent acinar secretory cell is identified by an arrowhead. X 55,000.

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18 A portion of a myoepithelial cell showing the following organelles: N, nucleus; M, mitochondria; R, ribosomes; T, bundles of myofilaments. Both surfaces of myoepithelial cell, one facing the basal lamina, the other juxtaposed to a secretory cell (C), show considerable pinocytosis. X 19,000.

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# The Effect of Estrogen on the Lipoprotein Lipase Activity of Rat Adipose Tissue

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ABSTRACT The effect of 17β-estradiol or progesterone administration on adipose tissue lipoprotein lipase activity was studied in male and ovariectomized female rats. Lipoprotein lipase activity was measured in acetone-ether-extracted preparations of adipose tissue with doubly labeled ($^{14}$C-fatty acid, $^{3}$H-glyceryl) chylomicron triglyceride as substrate. Administration of 17β-estradiol to male rats lowered adipose tissue lipoprotein lipase activity from 8.22±1.8 U/g (1 U = 1 μmol triglyceride hydrolyzed per h) to 4.96±0.5 U/g in the treated group. Ovariectomy increased adipose tissue lipoprotein lipase activity from 10.4±1.8 U/g in controls to 22.7±4.3 U/g. 17β-Estradiol administration to ovariectomized rats caused a marked fall in adipose tissue lipoprotein lipase activity: 17β-estradiol (2.5 μg/day) lowered the enzyme activity to 9.00±1.2 U/g, whereas 25 μg/day further decreased lipoprotein lipase activity to 3.2±0.6 U/g. Blood triglyceride levels increased from 0.8±0.05 μmol/ml in ovariectomized rats to 1.4±0.09 μmol/ml in 25 μg/day 17β-estradiol-treated rats. Progesterone administration did not affect adipose tissue lipoprotein lipase activity in either male or ovariectomized rats. Heart and lung lipoprotein lipase activity was unaffected by hormone treatment. We suggest that the rise in blood triglyceride concentrations, which accompanies high plasma estrogen levels, could be due to the marked inhibition of adipose tissue lipoprotein lipase activity.

## INTRODUCTION

There is now considerable evidence that plasma triglycerides are hydrolyzed to free fatty acids during their removal from blood. This process is catalyzed and regulated by lipoprotein lipase, an enzyme active in or near the capillary wall (1).

Lipemia of pregnancy, which is essentially a rise in plasma triglyceride concentration, is related to changes in the level of lipoprotein lipase activity in adipose tissue and mammary gland in the rat (2, 3). A few days before parturition, during the peak of hyperlipemia adipose tissue lipoprotein lipase is almost completely inhibited. Disappearance of the lipemia just before parturition is directly related to a marked rise in mammary gland lipoprotein lipase activity. In addition to pregnancy, increased plasma triglyceride levels have been reported in women on mixed contraceptives (4, 5); the lipemia seems to be caused by the estrogen component only (5).

There are marked changes in blood estrogen concentration during the estrous cycle in the rat (6). A comparison of lipoprotein lipase activity in adipose tissue of male with that of female rats showed that in males the activity level was almost constant, whereas wide fluctuations were observed in females.[1] This observation raised the possibility that female sex hormones might affect the activity of adipose tissue lipoprotein lipase.

## METHODS

*Animals.* Adult male and female Sprague-Dawley rats delivered by cesarean section were used in this study. The rats were purchased from Charles River Breeding Laboratories, Inc., Wilmington, Mass. The animals were caged individually and had free access to food (Purina Laboratory Chow, Ralston Purina Co., St. Louis, Mo.) and water.

*Hormone preparations.* 17β-Estradiol (Depo-estradiol cypionate) and progesterone (Depo-Provera [medroxyprogesterone acetate suspension U.S.P.]) where purchased from the Upjohn Co., Kalamazoo, Mich. Male rats were given the hormones by intraperitoneal injection once weekly for 8 wk: 17β-estradiol 50 μg, or progesterone 5 mg, in 0.1 ml saline. Hormone treatment of ovariectomized rats was started 2 wk after surgery. The rats were given 17β-estradiol or progesterone in 0.1 ml corn oil by subcutaneous

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TABLE I

*Effect of 17β-Estradiol* and Progesterone‡ on Adipose Tissue Lipoprotein Lipase Activity of Male Rats*

| Treatment§ | No. of animals‖ | Lipoprotein lipase activity¶ |
|---|---|---|
| | | U/g |
| None | 12 | 8.22±0.88 |
| 17β-Estradiol | 12 | 4.96±0.52** |
| Progesterone | 12 | 8.36±0.67 |

Results are mean±SE.

* 17β-Estradiol was injected i.p. 50 μg in 0.1 ml once weekly.

‡ Progesterone was injected i.p. 5.0 mg in 0.1 ml once weekly.

§ Hormones were administered for a period of 8 wk.

‖ Weight gain during the 8-wk period of treatment was 335±10.7 g in control, 320±24 g in progesterone-treated, and 290±7.8 g in 17β-estradiol-treated rats.

¶ 1 unit of lipoprotein lipase = 1 μmol triglyceride hydrolyzed per h. The activity rate for each tissue is the result of four separate determinations.

** Values significantly different from control ($P < 0.05$).

injection daily for 7 days. One group of animals received 2.5 μg 17β-estradiol, a second group 25 μg, a third group was given 5 mg progesterone; controls received 0.1 ml corn oil daily. The dosage of 17β-estradiol was chosen to be in the range administered by Chen and Meites (7) and lower than that given by Cairns and Constantinides (8).

*Lipoprotein lipase assay.* The animals were anesthetized with sodium pentobarbital (12.5 mg/rat) and exsanguinated through the heart. Ovariectomized rats were examined at autopsy to insure the complete removal of the ovaries. The uteri were dissected and weighed in order to check the effect of hormone treatment. Dried defatted powders of adipose tissue, heart, and lung were prepared and assayed for lipoprotein lipase activity as described previously (3, 9). Lipoprotein lipase activity was measured by the amount of rat chylomicron triglyceride hydrolyzed to fatty acids and glycerol with 1 U of activity = 1 μmol of triglyceride hydrolyzed per h (3, 9).

## RESULTS

*Effect of 17β-estradiol and progesterone on lipoprotein lipase activity in adipose tissue of male rats (Table I).* There was a marked decrease in lipoprotein lipase activity in epididymal adipose tissue of male rats treated with 17β-estradiol for a period of 8 wk. Progesterone treatment had no effect on adipose tissue lipoprotein lipase activity. The weight gain in the three groups of rats was not markedly different. Lipoprotein lipase activity in heart and lung of male rats was unaffected by estrogen or progesterone treatment.

*Effect of 17β-estradiol and progesterone on lipoprotein lipase activity in adipose tissue of ovariectomized rats (Table II).* The level of lipoprotein lipase activity in adipose tissue rose after ovariectomy from 10.40±1.8 U/g in control female unoperated rats to 22.72±4.3 U/g 3 wk after the operation. Plasma triglyceride concentration was in the normal range in both control and ovariectomized rats. Administration of 2.5 μg 17β-estradiol daily for a period of 7 days to ovariectomized rats decreased lipoprotein lipase activity in adipose tissue to levels similar to those in control unoperated animals. Increasing the dose of 17β-estradiol to 25 μg daily caused a further decrease in the level of adipose tissue lipoprotein lipase activity and a concomitant rise in plasma triglyceride concentration. Animals in the five different groups had a similar weight gain during the

TABLE II

*Effect of 17β-Estradiol and Progesterone on Lipoprotein Lipase Activity in Adipose Tissue of Ovariectomized Rats*

| Animals | Treatment* | No. of animals‡ | Lipoprotein lipase activity§ | Plasma triglyceride |
|---|---|---|---|---|
| | | | U/g | μmol/ml |
| Control | None | 6 | 10.40±1.8 | 0.85±0.05 |
| Ovariectomized | None | 9 | 22.72±4.3‖ | 0.80±0.05 |
| | 17β-Estradiol, 25 μg | 5 | 9.00±1.17¶ | — |
| | 17β-Estradiol, 25 μg | 5 | 3.20±0.60‖¶ | 1.40±0.09‖¶ |
| | Progesterone, 5 mg | 8 | 22.28±1.71 | — |

Results are mean±SE.

* 17β-Estradiol or progesterone were dispersed in corn oil and injected daily (0.1 ml subcutaneous) for a period of 7 days.

‡ Weight gain during the 7 days of treatment was 29±5 g in control, 32±4 g in ovariectomized, 24±3 and 21±2 g in 17β-estradiol-treated rats (2.5 and 25 μg/day, respectively), and 21±3.2 g in progesterone-treated rats.

§ The activity rate for each tissue is the result of four separate determinations.

‖ Values significantly different from control ($P = 0.01$).

¶ Values significantly different from ovariectomized ($P = 0.01$).



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hormone treatment period. Lipoprotein lipase activity in heart and lung was not affected by 17β-estradiol administration.

Heart lipoprotein lipase was 41.3±5.3 U/g in ovariectomized rats, 33.3±3.0 U/g in 2.5 μg and 43.9±3.3 U/g in 25 μg 17β-estradiol-treated animals, and 49.2±1.4 U/g in progesterone-treated rats. Lipoprotein lipase in the lung was 13.0±0.88 in control unoperated rats, 11.6±1.6, 12.00±2.54, 10.50±0.60, and 12.2±2.0 U/g in ovariectomized untreated, 2.5 μg and 25 μg 17β-estradiol- and progesterone-treated rats, respectively. Blood triglyceride levels were 0.85±0.05 μmol/ml in control unoperated rats, 0.80±0.05 μmol/ml in ovariectomized rats and 1.4±0.09 μmol/ml in ovariectomized rats treated with 25 μg/day 17β-estradiol. There was a sevenfold increase in the wet weight of the uterus after treatment of ovariectomized rats with either 2.5 or 25 μg 17β-estradiol daily for a period of 7 days; the uterus wet weight increased less than twofold after progesterone treatment.

## DISCUSSION

The effect of sex hormones on lipoprotein lipase activity has been limited to the study of the enzyme present in postheparin plasma (4, 5, 10, 11). These studies have been carried out mainly in the human (4, 5, 10, 11). Postheparin lipolytic activity is a mixture of enzymes containing lipoprotein lipase of extrahepatic origin and triglyceride lipase of hepatic origin (12). The two lipolytic activities, hepatic and extrahepatic, have different substrate specificity, triglyceride emulsions and lipoprotein-triglyceride, respectively, and respond differently to enzyme inhibition (12, 13). It has recently been shown that postheparin lipolytic activity of extrahepatic origin (12) is highest in postmenopausal women (13). In rats ovariectomy increases and estrogen administration decreases postheparin lipolytic activity (8). There is no information, however, on the effect of estrogen and progesterone on tissue levels of lipoprotein lipase in either man or other mammals. The present study shows that estrogen administration caused a marked decrease in the level of lipoprotein lipase activity in adipose tissue of male or ovariectomized rats (Tables I and II). There was a concomitant increase in plasma triglyceride concentration (Table II). Administration of progesterone did not affect the enzyme activity in adipose tissue of either male or ovariectomized rats.

Studies in vivo and in vitro in the rat have shown that lipoprotein lipase activity in adipose tissue is markedly decreased during fasting (14), lactation (2, 3), and in diabetes (14). The decrease in lipoprotein lipase activity after estrogen treatment was not caused by inadequate food intake since the rats in the different experimental groups (control, ovariectomized, and hormone treated) had a similar weight gain (Tables I and II).

We have recently shown that the very low activity of lipoprotein lipase in adipose tissue of lactating rats is the result of inhibition of enzyme synthesis by prolactin (15). We do not know at present whether the low adipose tissue lipoprotein lipase activity in estrogen-treated rats is caused directly by this hormone or whether it is mediated through the secretion of prolactin.

Estrogen administration to ovariectomized rats has been shown to increase both anterior pituitary and serum prolactin concentrations (7). The high levels of adipose tissue lipoprotein lipase activity in some of the control females and in pregnant rats during the first 2 wk of gestation (3) could be due to the low levels of both estrogen (6) and prolactin (16) during metestrus and diestrus and during the first 2 wk of gestation. Estrogen or progesterone treatment did not affect lipoprotein lipase activity in heart and lung.

We do not know at present what is the quantitative contribution of hepatic and extrahepatic tissues to the clearing of blood triglyceride. However, the presence of very low levels of lipase of extrahepatic origin and normal levels of hepatic lipase in postheparin plasma of patients with hyperchylomicronemia (13) indicates that the extrahepatic tissues play a major role in the clearing of blood triglyceride. This assumption agrees well with the data of Bragdon and Gordon, who showed that in the rat, 32% of chylomicron-triglyceride fatty acids were present in adipose tissue 10 min after intravenous injection of $^{14}$C-fatty acid-labeled chylomicrons (17). The decrease in adipose tissue lipoprotein lipase activity without the concomitant increase in enzyme activity in other tissues could lead to a rise in plasma triglyceride levels as a result of impaired clearing of blood triglyceride.

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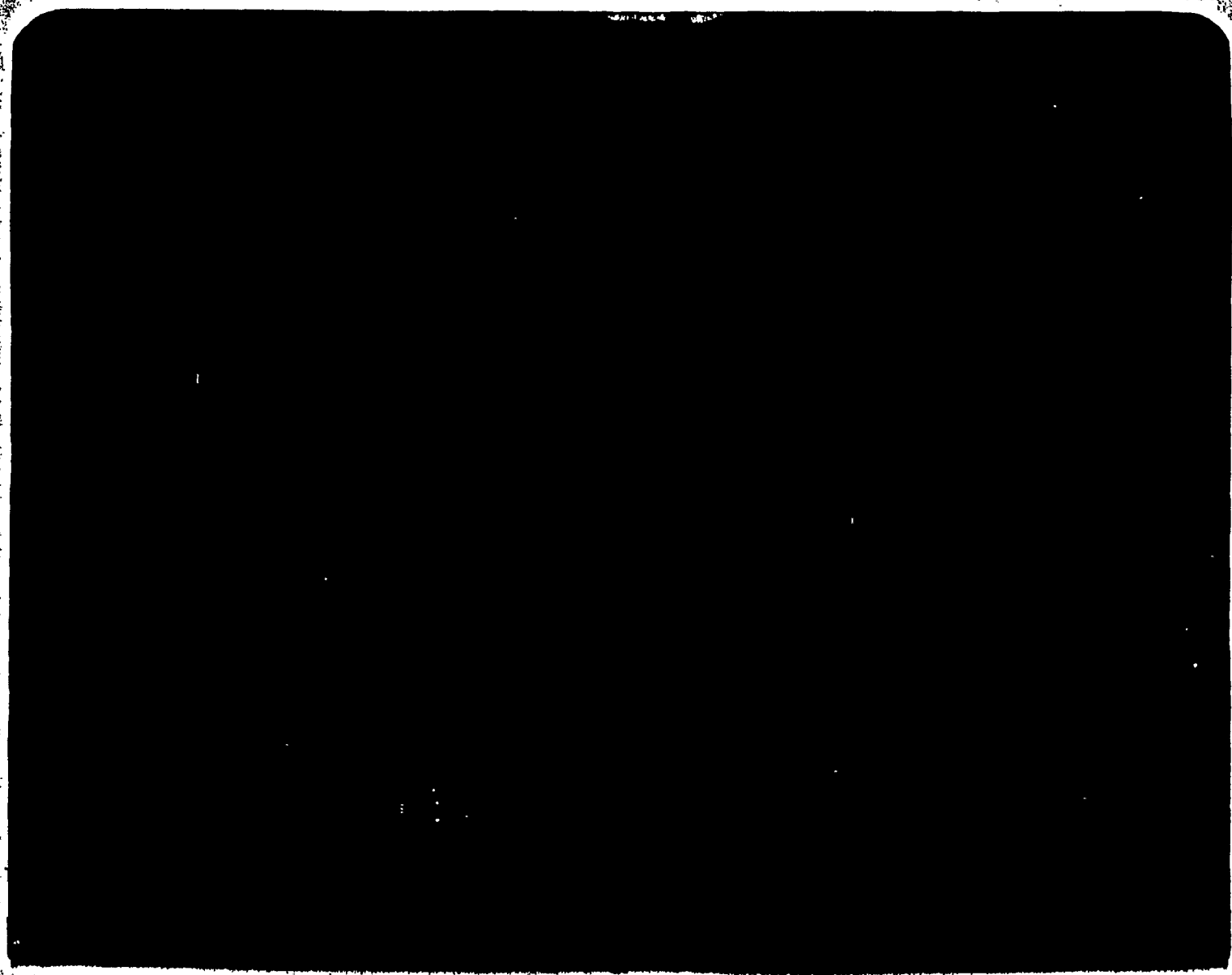
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